Food Microbiology and Food Safety
Research and Development

Sagar M. Goyal
Jennifer L. Cannon *Editors*

Viruses in Foods

Second Edition

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With love to:
Sarina, Milan, Sachin, Sapna, and Ezra

Preface to *Viruses in Foods,* 2nd Edition

Contamination of foods by enteric viruses is a major concern to public health and food safety. Foodborne viruses such as human norovirus and hepatitis A and E viruses are now well recognized for the number of foodborne illnesses and severity of disease they cause globally. In the final month this book was being prepared for publication, the World Health Organization's Foodborne Disease Burden Epidemiology Reference Group (FERG) released a report recognizing human noroviruses as the leading cause of foodborne illnesses and the fifth leading cause of foodborne deaths worldwide. Foods that are most commonly associated with viral foodborne illnesses are prepared foods contaminated at the point of food service and fresh produce and shellfish consumed raw. With the increasing demand for convenience foods and fresh produce, there is a need for up-to-date information on foodborne virus epidemiology, detection, prevention, and control.

Food Virology is a burgeoning field of emphasis for scientific research. Much progress has been made since the publication of the first edition of *Viruses in Foods* 10 years ago in 2006. Molecular detection assays for clinical disease are now well established, as are protocols for investigating potential foodborne virus outbreaks. Also, searchable international databases devoted to foodborne virus epidemiology have contributed greatly to the enhanced understanding of their prevalence and transmission dynamics. Many developments in foodborne virus detection, prevention, and control have also been made in recent years. The US Food and Drug Administration (FDA) and the European Standards Association (CEN) have published protocols, which have or are soon to become standard methods for virus detection in a variety of food types. In addition, *Codex Alimentarius* released guidelines for the control of foodborne viruses in foods and in environments where foods are produced, processed, and sold. Much of the basis for these publications has been laboratory research performed over the last decade. However, much work in the field is still needed and research has been hampered primarily due to our inability to routinely culture some of these viruses in the laboratory. Recent developments, however, may make it possible in the near future.

This second edition provides an up-to-date description of the major and some minor foodborne viruses infecting humans. It is unique in that a section is devoted to the study of foodborne virus epidemiology, including chapters that provide case reports of outbreaks occurring through different transmission routes so that lessons from the past can be reflected on as we move forward. It also includes a comprehensive section on methodologies for foodborne virus recovery and detection in foods, which can serve as a handbook for Food Virology practitioners. Lastly, the section on methodologies for prevention and control has been updated and expanded to uniquely include chapters on natural virucidal compounds in foods and risk assessment of foodborne viruses. The publication of this book is timely as much attention is now being paid to this important group of pathogens causing significant foodborne disease globally.

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CHAPTER 1

Food Virology: Advances and Needs

Charles P. Gerba

Food has been recognized as a vehicle for transmission of viruses for more than 100 years. Milk was identified as a vehicle for the transmission of poliomyelitis in 1914 (Jubb 1915). However, in the mid-1950s, hepatitis A transmission by shellfish was first reported in Sweden (Roos 1956) and then in the United States (Mason and McLean 1962). It was not until animal cell culture was developed, that the study of viruses in food could readily develop as a science. This allowed for the propagation of many of the enteric viruses and their detection in foods. Most of the early work centered on (1) shellfish because it was a known vehicle for hepatitis A transmission and (2) the use of treated wastewater for food crop irrigation. Dean Cliver was probably the first person that could call himself a food virologist. His career began in 1962 at the Food Research Institute, which started at the University of Chicago and later moved to the University of Wisconsin (Cliver 2010). His career saw the development of cell cultures to study viruses in foods and then to the age of molecular detection of viruses in foods. The focus of viruses in foods was originally on enteroviruses because they could readily be grown in cell cultures. Eventually rotavirus and hepatitis could be cultured, but it was not until molecular methods such as the polymerase chain reaction (PCR) were developed that the importance of norovirus and rotavirus in foodborne illness was fully appreciated. Today it is well documented in numerous epidemiological studies that noroviruses are the major cause of foodborne illness in the United States (Lopman et al. 2012). This has resulted in a rapid growth of the field of food virology in recent years.

As documented in this book great advances have been made in our understanding of foodborne viruses. However, much still needs to be done to control foodborne illnesses caused by the presence of viruses in our food supply. The lack of our ability to grow the human norovirus has led us to use surrogates for this virus. While this has provided many insights in the spread and potential control of noroviruses, uncertainty will always exist until routine methods become available to assess its infectivity. The high infectivity of viruses (low numbers to cause infection) and large amounts excreted in infected persons (10^{13} per day in an infected person) makes their control difficult.

While norovirus has received the greatest amount of attention as a cause of foodborne illness, other enteric viruses have recently been found to be common in foods, irrigation waters, and the food processing environment. In a large study of leafy greens in several European countries, human adenovirus

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was found to be the most commonly detected virus; it was detected in 20 % of 667 samples while noroviruses were found in only 3 % of samples (Kokkinos et al. 2012). In addition, most of the contamination occurred previous to processing (i.e. in irrigation waters or workers hands). In a study of berry production in Europe, adenoviruses were again found to be the most common virus on the berries and in the production chain (Maunula et al. 2013). This indicates that contamination of foods by viruses other than norovirus may be more common, but their impact on human health remains uncertain.

Risk assessment has been a vital tool in understanding the significance of detecting viruses in foods and the potential effectiveness of different interventions. It has shown us that certain segments of our population are at increased risk of serious illness and mortality from acute diseases. However, recent long term studies on the impact of chronic illness after waterborne outbreaks of enteric pathogens have demonstrated effects that last years and perhaps a lifetime (Moist et al. 2009). These impacts of foodborne viruses need to be better quantified and understood to appreciate the long term effects of foodborne viral illness on populations. In addition, risk assessment of foods needs to move beyond agents that cause gastroenteritis alone. Molecular methods have been increasingly used to demonstrate the role of enteroviruses and adenoviruses in drinking water outbreaks (La Rosa et al. 2012). These viruses cause a wide variety of illnesses; from neurological disorders to heart disease. Their presence in foods indicates a risk to populations and this needs to be better articulated in the food safety agenda. While their role in foodborne transmission may be lower, the potential outcomes on human health may be greater.

Risk assessment also needs to be brought to bear on the issue of needed levels of virus reduction by disinfectants. Do we really need a 3–5 log reduction of infectious virus quantities in laboratory tests on disinfectants to reduce the risk? In the drinking water guidelines for water treatment a 4 log reduction in infectivity for viruses is required based on the likely concentration of viruses in the untreated water (Regli et al. 1991). In drinking water a goal of a risk of 1:10,000 per year of acquiring an infection is recommended. Defining goals would greatly aid the design of disinfectants and interventions in the food industry. Application of virus surrogates would also be useful in quantifying spread and the impact of interventions on reduction in the risk of infection (Sinclair et al. 2012). The use of harmless coliphages to better understand virus movement in households, restaurants and food processing may be better risk models to define control strategies.

Application of disinfectants or other anti-microbials for the control of viruses in foods is challenging because of the inherent demand placed by the organic nature of the foods on the delivery of effective doses. Chlorine and other oxidizers combine with debris and the organic compounds in foods making increased doses necessary. The rough surface of some produce such as strawberries also furnishes locations which the anti-microbials cannot reach. One approach to overcome these limitations is the use of natural plant

antimicrobials, such as oregano. In the laboratory these substances appear very effective, but cost and effectiveness in practice still need to be assessed.

Many challenges remain in the application of molecular methods for the study of viruses in foods and methods to control them. While molecular methods have helped advance the field in increasing the types of viruses in foods and water that can be detected, they still cannot with certainty determine if a virus is still infectious or not (Rodriguez et al. 2009). The mechanisms of virus inactivation vary among the types of viruses (RNA or DNA) and the type of antiviral agent. If the mechanism of viral inactivation is known, the molecular method may be modified to address virus viability. However, molecular methods are limited to small assay volumes, thus affecting both the sensitivity of these methods and our ability to effectively determine significant virus reduction in foods and water by treatment processes. Given the high infectivity of viruses (small doses to needed to case infection), it is important to be able to have methods that can detect low levels of viruses in food.

In summary, while major advances have been made in the last decade in understanding the importance of foods in the transmission of viruses, much still needs to be done to totally understand their impact and to develop methods for their control.

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CHAPTER 2

Human and Animal Viruses in Food (Including Taxonomy of Enteric Viruses)

Gail E. Greening and Jennifer L. Cannon

1. INTRODUCTION

In recent years, there has been an increase in the incidence of foodborne diseases worldwide, with viruses now being recognized as a major cause of these illnesses. The most common viruses implicated in foodborne disease are enteric viruses, which are found in the human gastrointestinal tract, excreted in human feces and transmitted by the fecal-oral route. Many different viruses are found in the gastrointestinal tract but not all are recognized as foodborne pathogens. The diseases caused by enteric viruses fall into three main types: gastroenteritis, enterically transmitted hepatitis, and illnesses that can affect other parts of the body such as the eye, the respiratory system and the central nervous system leading to conjunctivitis, poliomyelitis, meningitis and encephalitis. Viral pathogens excreted in human feces include noroviruses, sapoviruses, enteroviruses, adenoviruses, hepatitis A virus (HAV), hepatitis E virus (HEV), rotaviruses, and astroviruses. Most of these viruses have been associated with foodborne disease outbreaks. Noroviruses and HAV are commonly identified as foodborne causes of gastroenteritis and acute hepatitis, respectively.

Other human- and animal-derived viruses, which have the potential to be transmitted by food, do not always infect the gastrointestinal tract. For example, HAV and HEV infect the liver; enterovirus, poliovirus, Nipah virus and tick-borne encephalitis virus infect the nervous system; and the SARS (severe acute respiratory syndrome) and the MERS (Middle East respiratory syndrome) coronaviruses and avian influenza virus H5N1 infect the respiratory system (FAO/WHO 2008). Members of several virus families (including *Birnaviridae*, *Circoviridae*, *Papillomaviridae*, *Parvoviridae and Polyomaviridae*) can survive for a prolonged period outside the host, a suitable characteristic for foodborne transmission (Koopmans et al. 2008).

Five of the enteric viruses, namely norovirus, HAV, sapovirus, rotavirus and astrovirus, are included in the 31 major foodborne pathogens identified by the Centers for Disease Control and Prevention (CDC) (Scallan et al. 2011). Of the estimated 37 million foodborne illnesses that occur annually in the United States, viruses cause 59 % of illnesses with noroviruses being the greatest

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contributor, causing an estimated 5.5 million cases per year (Scallan et al. 2011). The European Food Safety Authority (EFSA) identified norovirus (NoV) and HAV as the major foodborne viruses of public health significance. The HEV was also included because of its high prevalence in pigs and the potential for zoonotic transmission (EFSA 2011). Several virus-commodity combinations were identified as high priority for implementation of prevention and control measures:

- NoV and HAV in bivalve molluscan shellfish
- NoV and HAV in fresh produce
- NoV and HAV in prepared (ready-to-eat) foods
- Rotaviruses in water for food preparation
- Emerging viruses in selected commodities

With the exception of shellfish, which are most often contaminated by sewage infiltration of harvesting waters, food handlers are the most significant source of viral foodborne illnesses. Foods most often implicated include salads, fresh produce and bakery or delicatessen items that are prepared or handled raw or after the foods have been cooked. The EFSA now recommends a focus on measures such as encouraging good hand hygiene and sanitation to prevent viral contamination of foods rather than virus removal or inactivation after foods have become contaminated (EFSA 2011).

All enteric viruses except the adenoviruses contain RNA, have a protein capsid that protects the nucleic acid, and are non-enveloped. In the environment and in food, the enteric viruses are inert particles and do not replicate or metabolize because, like all other viruses, they are obligate pathogens and require living cells to multiply. Cell cultures are generally used for the growth and analysis of culturable viruses. Using culture methods, infectious viruses can be identified through their ability to produce cell changes or cytopathic effects (CPE), or through expression of viral antigens that may be detected serologically. The advantage of culture-based methodology is that it can be either quantitative or qualitative and produces unambiguous results with respect to virus presence and infectivity. Many of the enteric viruses such as astroviruses, enteric adenoviruses, HAV and rotaviruses are fastidious in their *in vitro* growth requirements but can still be grown in cell cultures with some effort. Despite numerous attempts to grow human norovirus (HuNoV) *in vitro* in traditional cell lines, primary cell cultures, or differentiated tissues grown in three-dimensional cultures (Duizer et al. 2004; Lay et al. 2010; Papafragkou et al. 2013), they have until recently remained recalitrant. Low-level HuNoV infection in cultured B-cells was recently reported (Jones et al. 2014). Such findings are exciting and promising, but the model awaits optimization and validation before it can be routinely applied. In addition, no animal model for Human NoV infection has been validated, although recent reports on replication of this virus in immunocompromised mice and gnotobiotic animals suggest that this task is not out of reach (Tan and Jiang 2010; Bok et al. 2011). In the meantime, surrogate viruses that are closely related to HuNoV and are

easily cultured in the laboratory, are used to conduct studies on the disinfection and environmental persistence of HuNoV.

Until the introduction of molecular methods, enteric viruses were mainly identified by electron microscopy (EM) including solid phase immune electron microscopy (SPIEM). The SPIEM is more sensitive than direct EM because, in the presence of specific antibodies, the virus particles are aggregated together making them more easily distinguishable from the background matrix. Many of the 'small round viruses', which include astroviruses, noroviruses, sapoviruses and parvoviruses, were first discovered through the use of EM. Molecular methods, especially real-time quantitative PCR, are now the most commonly used techniques for the identification of enteric viruses in foods. Other methods for identification of enteric viruses in human specimens include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and culture-PCR, which is a combination of cell culture and PCR methods. The latter technique detects only the infectious virus and is preferable to direct PCR, which currently detects both infectious and non-infectious viruses.

The enteric viruses are generally resistant to environmental stressors, including moderate heat and acid extremes. Most resist freezing and drying and are stable in the presence of organic solvents. It is not clear whether pasteurization at 60 °C for 30 min inactivates all enteric viruses in all food types. Many enteric viruses show resistance to high hydrostatic pressure, which is now widely used as a food processing treatment for shellfish, jams, jellies and dairy products (Kingsley 2013). The resistance of enteric viruses to environmental stressors allows them to survive both the acidic environment of the mammalian gastrointestinal tract and also the proteolytic and alkaline activities of the duodenum, so that infection can be established in the small intestine. Thus, enteric viruses can survive in acidic foods, marinated and pickled foods, frozen foods and lightly cooked foods such as gently steamed shellfish. Most enteric viruses are believed to have a low infectious dose of 10–100 particles or possibly even less. Hence, although they do not multiply in food, enough infectious viruses may survive in food to cause infection.

Enteric viruses have been shown to retain infectivity in shellfish and in fresh, estuarine and marine waters for several weeks to months at 4 °C (Bae and Schwab 2008). The length of virus survival appears to be inversely related to temperature. They may survive longer if attached to particulate matter or sediments, where they can present a greater potential risk to human health (Jaykus et al. 1994). Numerous studies have been published in recent years assessing the fate of foodborne viruses in the environment and their transfer to foods during preparation and handling (Kotwal and Cannon 2014).

Viral contamination of foods can occur before, during or after harvest at any stage in the food harvesting, processing, distribution and retail chain, or by food handlers in the home or restaurant setting. The key factors influencing the risk of contamination of fresh produce are water quality and food worker hand hygiene. Thus sewage contamination and poor hygiene practices play a major role in the contamination of produce.

Numerous opportunities exist for both pre- and post-harvest viral contamination of food. The quality of shellfish growing waters is important; pre-harvest virus contamination occurs when filter-feeding bivalve shellfish grow in waters contaminated with sewage or fecal material. Shellfish filter between four and 20 L of water every hour, sieving out and accumulating food particles, including bacteria, viruses and heavy metals. Feeding rates depend upon water temperature and salinity and availability of food and particulate matter. Bacteria and viruses become trapped in the mucus of the gills, which is then pushed into the digestive gland where viruses concentrate. Shellfish can accumulate high concentrations of viruses within a few hours e.g., they may contain virus levels that are 100–1000 times greater than in the surrounding waters.

Different species of oysters take up enteric viruses at different rates (Nappier et al. 2008, 2010). In winter, the shellfish are physiologically less active and do not accumulate viruses as fast as in warmer seasons. In clean waters, shellfish depurate or cleanse themselves of bacteria and particulate matter. However, studies have shown that depuration does not remove viruses efficiently and that there is no correlation between the removal of bacteria and viruses (Lees 2000). This was demonstrated in a large hepatitis A outbreak in Australia where oysters were depurated for 36 h before consumption but still retained infectious HAV (Conaty et al. 2000). Recent laboratory-based depuration experiments have shown that poliovirus, HAV and norovirus are eliminated at different rates from Pacific oysters over a 48 h period; most of the poliovirus was inactivated but HAV remained infectious over the 48 h period (McLeod et al. 2009). Human noroviruses are particularly recalcitrant to removal by depuration due to specific carbohydrate moieties expressed in the digestive diverticula of some filter-feeding bivalves that bind and retain the viruses (Le Guyader 2006; Maalouf et al. 2010; Tian et al. 2007).

The globalization of the food supply means that the source of fresh produce may not always be known and the quality may not always be controlled. Although it is presumed that fresh produce is "clean, green and healthy", it may not be so, especially when it is imported from countries where general hygiene practices do not meet international standards. In recent years, there have been numerous outbreaks of HuNoV and HAV associated with contaminated fresh produce imported into Europe, Australia and North America (Anonymous 2013; Carvalho et al. 2012; Cotterelle et al. 2005; Donnan et al. 2012; Ethelberg et al. 2010; Falkenhorst et al. 2005; Fournet et al. 2012; Korsager et al. 2005; Le Guyader et al. 2004; Made et al. 2013; Maunula et al. 2009). Confirming the sources of contamination during these outbreaks has proven to be very difficult. In some cases, more than one pathogen or strain of virus was associated with the outbreak, suggesting that sewage-impacted irrigation or produce-wash water may have been involved.

Fresh produce may become contaminated with disease-causing enteric viruses if irrigated with or washed in water containing human fecal material or if handled by field-workers or food-handlers with poor hygiene practices. Foods at the greatest risk of virus contamination at the pre-harvest, harvest or

post-harvest processing stage are shellfish, soft berry fruits, herbs and salad greens. Foods that are subject to much handling and are subsequently consumed cold or uncooked are at risk of contamination from food handlers. Such foods include bread and bakery goods, lightly cooked or raw shellfish, sandwiches, salads, herbs, fresh fruits, cold meats and cold desserts. It is probable that the current trend for the consumption of raw or lightly cooked ready-to-eat (RTE) foods, especially salads and sandwiches, has increased the risk of foodborne viral disease. Poor food handling was shown to be a key risk factor in the transmission of HuNoV in New Zealand and of HuNoV and rotaviruses in the Netherlands (de Wit et al. 2003; Thornley et al. 2013). In the U.S., infected food handlers are the source of over 50 % of all foodborne HuNoV outbreaks, and may even play a contributing role in over 80 % of them (Hall et al. 2012).

Viruses are frequently host specific, preferring to grow in the tissue of one species rather than a range of species. Both animal and human strains exist in all of the enteric viral genera. A key question still to be answered is whether animal viruses can infect humans and vice versa. The pathogenic strains of astrovirus, adenovirus and enterovirus that infect animals appear to be distinct from those infecting humans. Thus, although noroviruses have been isolated from animal feces and antibodies to animal noroviruses have been detected in humans, so far they have not been implicated in human disease.

Zoonotic infections are generally not transmitted by food. However, the risk of zoonotic viral disease from meat products contaminated with animal viruses has been identified in some countries, with tick-borne encephalitis virus (TBE) and hepatitis E virus (HEV) being two examples. The HEV is possibly the first virus reported to cause zoonotic foodborne viral disease. Non-viral infectious proteinaceous agents or prions, that cause diseases such as bovine spongiform encephalopathy (BSE), scrapie and Creuzfeldt-Jacob disease, also transmit disease from animals to humans via the foodborne route but are not discussed in this review.

As a result of the advances in methodology for detection of viruses in foods, the extent and role of viruses in foodborne disease has been clarified in recent years. New molecular methods, including real-time quantitative PCR for the detection of non-culturable or difficult to culture viruses, have shown their frequent presence in the environment and foods, especially shellfish. These methods have also allowed investigation of virus responses to environmental stressors and have contributed to increased knowledge of enteric virus behavior in the foods and the environment.

2. HEPATITIS A VIRUS

2.1. Distribution and Transmission

Several different viruses cause hepatitis but only two, namely HAV and HEV, are transmitted by the fecal-oral route. The hepatitis viruses are so named because they infect the liver. These viruses do not share phylogenetic or

morphological similarities and each of the five different hepatitis viruses is classified in distinct virus families. The HAV causes hepatitis A, a severe food and waterborne disease primarily transmitted by the fecal-oral route, which was formerly known as infectious hepatitis or jaundice. Hepatitis A infection occurs worldwide and is especially common in developing countries where over 90–100 % of children are infected by 6 years of age (Cliver 1997; Cromeans et al. 2001; Franco et al. 2012). The infection is often asymptomatic in children.

In recent years, the incidence of HAV infection in many countries has decreased as sewage treatment and hygiene practices have improved, but this has also led to an overall lowering of immunity in these populations with consequent increase in susceptibility to the disease. As a result, there is an increasing risk of contracting HAV infection from fresh foods imported from regions of the world where HAV is endemic and general hygiene standards are poor. Hepatitis A is a serious foodborne infection and hence is a notifiable disease in most of the developed countries. In the United States, HAV is reported as the most common cause of hepatitis with an estimated death rate of 2.4 % for laboratory confirmed cases (Scallan et al. 2011). The majority of illnesses (41 %) are related to travel but approximately 1500 cases (7 %) each year in the US are due to domestically acquired foodborne illnesses. In 2010, 1670 illnesses were reported to the CDC in the US. Taking into account asymptomatic infections and underreporting, it is estimated that there were 17,000 cases, an 88 % decline from the past decade (CDC 2010).

No seasonal distribution of HAV has been observed, with infection occurring throughout the year, but the disease has been reported to have a cyclic occurrence in endemic areas. This cyclic pattern has been observed in the United States, particularly among low socio-economic Native American and Hispanic populations. Large increases in HAV infections occur approximately every 10 years when a new cohort of susceptible children within these communities reach an age when they can become clinically symptomatic. However, the main transmission route is generally believed to be person-to-person rather than foodborne (Cromeans et al. 2001; Fiore 2004).

2.2. Taxonomy and Morphology

The HAV is a 27–32 nm non-enveloped, positive-sense, single-stranded RNA virus with a 7.5 kb genome, icosohedral capsid symmetry, and a buoyant density in cesium chloride of 1.33–1.34 g/ml. The virus is classified in the *Picornaviridae* family in its own distinct genus, *Hepatovirus* (Table 2.1). Hepatoviruses do not exhibit the "canyon-like" cellular receptor binding sites on their capsid surface causing differences in cell interaction, which sets them apart from other picornaviruses (Cristina and Costa-Mattioli 2007). The single species of HAV was previously classified into seven genotypes based on partial sequences of VP1 and VP3 genes that code for surface proteins (Robertson et al. 1992; Robertson et al. 1991). However, full-length VP1 sequencing has now revealed similarities between genogroup II and IV strains, sub-grouping

Table 2.1 Characteristics of foodborne viruses

Virus genus or species	*Family*	*Nucleic acid type*	*Envelope*	*Morphology/ symmetry*	*Size of virion (nm)*	*Culturable*[a]	*Genome size (Kb)*	*Disease*
Adenovirus	Adenoviridae	dsDNA	N	Icosohedral	80–110	Y[a]	28–45	Respiratory, eye and gastroenteritis infection
Aichivirus	Picornaviridae	(+) ss RNA	N	Icosahedral	20–40	Y	8.2	Gastroenteritis
Astrovirus	Astroviridae	(+) ssRNA	N	Icosohedral	28–30	Y[a]	6.8–7.8	Gastroenteritis
Coronavirus	Coronaviridae	(+) ssRNA	Y	Helical	80–220	Y[a]	27–32	Gastroenteritis, respiratory infections
Norovirus	Caliciviridae	(+) ssRNA	N	Icosohedral	28–35	N	7.4–7.7	Epidemic gastroenteritis
Sapovirus	Caliciviridae	(+) ssRNA	N	Icosohedral	30–38	N	7.1–7.7	Gastroenteritis
Hepatovirus: Hepatitis A virus	Picornaviridae	(+) ssRNA	N	Icosohedral	27–32	Y[a]	7.5	Inflammation of liver, hepatitis
Hepevirus: Hepatitis E virus	Hepeviridae	(+) ssRNA	N	Icosohedral	27–34	Y	7.5	Inflammation of liver, hepatitis
Rotavirus	Reoviridae	dsRNA, segmented	N	Icosahedral	60–80	Y	16–27	Gastroenteritis
Enterovirus	Picornaviridae	(+) ssRNA	N	Icosohedral	28–30	Y[a]	7.2–8.4	Poliomyelitis, respiratory infection, meningitis, encephalitis
Parvovirus	Parvoviridae	ssDNA	N	Icosohedral	18–26	N	5	Gastroenteritis
Picobirnavirus	Picobirnaviridae	dsRNA, segmented	N	Icosohedral	33–41	N	3.8–4.5	Gastroenteritis in animals and ? humans
Tick-borne encephalitis virus	Flaviviridae	(+) ssRNA	Y	Icosohedral	45–60	Y	9.5–12.5	Tick-borne encephalitis via milk
Torovirus	Coronaviridae	(+) ssRNA	Y	Helical	100–150	Y	27–32	Gastroenteritis in animals and ? humans

[a]Not all strains within the genus are culturable, wild-type strains are often difficult to culture.

of genogroup II and the elimination of genogroup VII. Genogroups I-III contain A and B sub-genogroups (Hollinger and Emerson 2007).

The HAV genotypes are phylogenetically distinct and have different preferred hosts. For example, genotypes, I-III and IV-VI infect humans and simians, respectively. Human genotypes infect all species of primates including humans, chimpanzees, owl monkeys and marmosets, whereas simian genotypes infect non-human primates including green monkeys and cynomolgus monkeys (Aggarwal and Naik 2008). Characterization of these genotypes has been useful in outbreak investigations for tracing infection sources; virus strains within these genotypes have over 85 % genetic similarity (Cromeans et al. 2001; Niu et al. 1992). There is a single serotype for all HAV genotypes. Mutation rates for HAV are much lower than those reported for other picornaviruses, due mostly to constraints of the viral capsid being unfavorable to amino acid substitutions (Cristina and Costa-Mattioli 2007).

2.3. Growth and Biological Properties

The HAV can be cultured in several different primate cell lines including African green monkey kidney cells (BSC-1), fetal rhesus monkey kidney cells (FRhK-4 and FRhK-6) and human fibroblasts (HF), but wild type strains are difficult to culture and generally do not produce CPE in cell cultures. Immunofluorescence is often used for detection of HAV antigen in infected cells because of the lack of CPE. The virus is usually slow-growing and the yield in cell cultures is low compared to most other picornaviruses. Consequently it is difficult to identify the virus in clinical, food or environmental sources by culture alone. Under normal conditions, the virus requires 3 weeks for *in vitro* growth. Laboratory-adapted strains such as HM 175 are able to produce CPE and have been used extensively in research studies. These strains require less time for *in vitro* growth and produce visible CPE or plaques. Molecular techniques, including culture-PCR, have become the method of choice for detection of HAV in non-human samples. In humans, clinical diagnosis is usually based on the patient's immune response. The HAV antigens are conserved and antibodies are generated against a single antigenic site comprised of amino acid residues of VP3 and VP1 proteins on the virus surface.

The virus is very stable, showing high resistance to chemical and physical agents such as drying, heat, low pH and solvents and has been shown to survive for over 3 months in the environment, including seawater and marine sediments (Sobsey et al. 1988). The virus exhibits greater thermal stability at high temperatures when compared to norovirus surrogates. The resistance of HAV to heat is greater in foods and shellfish due to reduced heat transfer and protection by the shellfish tissue. Under refrigeration and freezing conditions the virus remains intact and infectious for several years. It is also resistant to drying, remaining infectious for over 1 month at 25 °C and 42 % humidity, and shows even greater resistance at low humidity and low temperatures. The survival of HAV on fresh and semi-dried tomato (SDT) surfaces was investigated

following a recent outbreak of HAV. Results showed that inactivation of HAV on SDT using chemicals or heat was difficult and that it was important to inactivate HAV prior to or during the manufacture of SDT because, following manufacture, any HAV remaining on SDT could retain infectivity at both room temperature and at 5 °C for periods beyond the shelf life of the product (Greening and Hewitt 2012).

In other studies, 2–5 $\log_{10}$ of virus were inactivated following exposure to 70 % alcohol for 3 min; however, it was resistant to several preservatives and solvents including chloroform, Freon, Arklone and 20 % ether. In addition, HAV was not inactivated by 300 mg/L perchloroacetic acid or 1 g/L chloramine at 20 °C for 15 min (Hollinger and Emerson 2007) although it was susceptible to formalin (3 % for 5 min or 8 % for 1 min at 25 °C), iodine (3 mg/L for 5 min) and ß-propiolactone (0.03 % for 72 h at 4 °C) (Hollinger and Emerson 2007). The virus is stable at pH 1.0 and survives acid marination at pH 3.75 in mussels for at least 4 weeks (Hewitt and Greening 2004; Hollinger and Emerson 2007). Gamma irradiation is not effective for inactivation of HAV on fresh fruits and vegetables and high doses of ultraviolet radiation are required for inactivation (Hollinger and Emerson 2007). Hydrostatic pressure, now used as an isothermal preservation method for perishable foods, inactivated HAV following 5 min exposure at 400 MPa (Kingsley 2013). Free chlorine concentrations of less than 10 ppm inactivate HAV at room temperature when sufficient exposure times are provided (5–15 min) and organic solids contribute minimally to chlorine demand (Hollinger and Emerson 2007). Like many enteric viruses, fecal material and virus aggregation provide protection to HAV against chemical and physical assaults. Overall HAV exhibits greater resistance to stressors than other picornaviruses.

2.4. Infection and Disease

The HAV infects the epithelial cells of small intestine and hepatocytes causing elevation of liver enzymes and inflammation of the liver. Cytotoxic T-cell immune response destroys infected liver cells releasing the virus particles into the bile duct from where they are excreted in the feces. The virus is believed to initially enter the liver via the bloodstream and it is not clear if intestinal replication occurs. The disease has an incubation period of 2–6 weeks with an average of 28 days. Initially the symptoms are non-specific and include fever, headache, fatigue, anorexia, dark urine, light stools, and nausea and vomiting with occasional diarrhea. One to two weeks later, characteristic symptoms of hepatitis such as viremia and jaundice appear. Peak infectivity occurs in 2 weeks preceding the onset of jaundice and the virus is present in the blood at 2–4 weeks. The virus is shed in large numbers (>10^6 particles/g) in feces from the latter 2 weeks of the incubation period and for up to 5 weeks. Jaundice is usually evident from week 4–7 and virus shedding generally continues throughout this period.

Diagnosis is based on the detection of anti-HAV IgM antibody, which can be detected before the onset of symptoms but becomes undetectable within

6 months of recovery. Acute hepatitis is usually self-limiting, but overall debility lasting several weeks is common and relapses may occur. The HAV has not been associated with development of chronic liver disease but on rare occasions, fulminant disease that results in death may occur. Because the onset of symptoms occurs several weeks after infection, it is rare to have the suspected food available for analysis. A killed vaccine that provides long-lasting immunity has been commercially available since 1995 and is commonly given to travelers at high risk. It is now a part of the childhood vaccination schedule in the US, as recommended by the CDC. This vaccine could be used in the food industry to immunize food workers to reduce the risk of food contamination by these workers.

2.5. Foodborne Disease

The HAV has been associated with many outbreaks of foodborne disease; contamination generally occurring either pre-harvest or during food handling. There are a number of documented outbreaks of disease resulting from consumption of HAV-contaminated shellfish, the largest of which occurred in China in 1988 when approximately 300,000 people were infected following consumption of partially cooked clams harvested from a growing area impacted by raw sewage (Halliday et al. 1991). Other shellfish-associated outbreaks include oysters in Australia (Conaty et al. 2000), oysters in Brazil (Coelho et al. 2003), mussels in Italy (Croci et al. 2000) and clams in Spain (Bosch et al. 2001). In most of these outbreaks sewage was generally the source of pollution. Contamination of shellfish with HAV is still common in Italy, Spain and other European countries. Pre-harvest contamination of fruits and vegetables, including strawberries (Niu et al. 1992), raspberries (Ramsay and Upton 1989; Reid and Robinson 1987), blueberries (Calder et al. 2003), frozen pomegranate seeds (Anonymous 2013), lettuce (Pebody et al. 1998), semi-dried tomatoes (Donnan et al. 2012; Petrignani et al. 2010) and green onions (CDC 2003), has also been reported and has resulted in outbreaks of disease in countries such as Finland, the Netherlands, the United States, Australia and New Zealand, where populations have low or no immunity to the disease (Calder et al. 2003; Donnan et al. 2012; Pebody et al. 1998). The source of contamination in these outbreaks was reported to be either infected food handlers or contaminated irrigation waters.

The other main source of HAV infection is from food handlers and food processors.Since HAV is shed before symptoms become apparent (as much as $>10^6$ infectious virus particles can be excreted per gram of feces), HAV-infected produce harvesters and food handlers, without knowing, may become a source of contamination. In areas with poor hygiene practices, this can present a very high risk to human health. Foodborne outbreaks of HAV are relatively uncommon in developing countries where there are high levels of immunity in the local population, but tourists in these regions can be susceptible if they are not vaccinated.

3. HEPATITIS E VIRUS

3.1. Distribution and Transmission

The hepatitis E virus (HEV) is a major etiologic agent of enterically transmitted hepatitis in humans and domestic swine worldwide. In endemic regions of the world, Global Burden of Disease estimates are 20 million infections, 3.4 million symptomatic illnesses, 70,000 deaths and 3000 stillbirths (Dalton et al. 2013). In India, mortality estimates among pregnant women reach 1000 per year (Dalton et al. 2013). Waterborne outbreaks and secondary person-to-person spread involving thousands and even tens of thousands of persons have been reported in China, India, and Africa (CDC 2013; Kamar et al. 2012). In developed countries, sources of HEV infection in humans are largely unrecognized but evidence for zoonotic and foodborne transmission is emerging. In fact, HEV is now regarded as a significant emerging zoonotic and potential foodborne pathogen by the European Food Safety Authority (EFSA 2011). Seroprevalence rates of 16–29 %, and as high as 52 % in a hyperendemic region of Southern France, have been reported among adults in Europe. Discrepancies between seroprevalence and clinically confirmed cases suggest that asymptomatic infections and underreporting are common.

3.2. Taxonomy and Morphology

The HEV was first isolated and identified by Balayan et al. (1983) in acute and convalescent specimens collected from a case of non-A, non-B hepatitis (Balayan et al. 1983). The HEV is 27–34 nm, non-enveloped virus with positive-sense, single-stranded RNA (linear genome of 7.2 kb) (Table 2.1). The capsid symmetry is icosohedral and the buoyant density in potassium tartrate—glycerol gradient is 1.29 g/ml. Virus particles isolated from serum associate with lipids and therefore have a lower density (1.15–1.16 g/ml) in sucrose fractions than do HEV isolated from feces (1.27–1.28 g/ml) (Kamar et al. 2012). The HEV was originally classified in the *Caliciviridae* family because of similarities in structural morphology and genome organization but was later reclassified under *Togaviridae* because of similarities between the replicative enzymes of HEV and the togaviruses. Until very recently, the International Committee on Taxonomy of Viruses (ICTV) classification placed HEV under a new family *Hepeviridae* under the single genus *Hepevirus* (Van Regenmortel 2000). The family has since been divided into two genera; Orthohepevirus, which infects mammalian and avian species and Piscihepevirus, which includes only the cutthroat trout virus (Smith et al. 2014). While there are four groups of orthohepevirus (groups A-D), only group A is known to infect humans (Smith et al. 2014). Within group A, there are four HEV genotypes (HEV1-HEV4) that infect humans and other mammals but only a single serotype (Dalton et al. 2013).

The HEV1, HEV2, HEV3 and HEV4 can be further divided into at least 5, 2, 3 and 7 subgenotypes, respectively (Kamar et al. 2012). The HEV1 and

HEV2 primarily cause disease in areas of the world where water quality and sanitation are inadequate; HEV1 primarily circulates in Southeast Asia and Africa while HEV2 has been detected in Mexico and parts of Africa. The HEV3 is detected worldwide but HEV4 has primarily been detected in Southeast Asia and recently in Europe. Historical evidence suggests HEV1 and/or HEV2 circulated in Europe and the United States at the turn of the twentieth century, but disappeared with water and sanitation improvements after World War II (Dalton et al. 2013).

The HEV3 primarily circulates in swine and wild boars worldwide but occurs mainly in China and Japan. Swine and human strains of HEV share close sequence homology. Both HEV3 and HEV4 strains have been detected in retail pork products (Berto et al. 2013; Berto et al. 2012; Colson et al. 2010) and epidemiological investigations strongly point to human infections caused by consumption of pork products and game meat (Li et al. 2005; Masuda et al. 2005; Matsubayashi et al. 2008; Matsuda et al. 2003) and deer (Mushahwar 2008; Takahashi et al. 2003; Tei et al. 2003). Other mammals such as rats, bats, rabbits and ferrets are known to harbor HEV, but no cases of human disease have been associated with these animals. Contamination of water, shellfish and fresh produce with HEV has also been documented, suggesting the occurrence of non-zoonotic sources of foodborne infections and/or water and crop contamination with swine or other animal waste (Meng 2011). Blood transfusions and organ transplants are another significant source of HEV infection in the developed world (Mushahwar 2008). Secondary person-to-person transmission is relatively uncommon and is estimated at 0.7–8.0 % (Cromeans et al. 2001).

3.3. Growth and Biological Properties

In vitro growth in cell culture has only recently been possible for HEV1, HEV3 and HEV4. Hepatic carcinoma (PRC/PRF/5) and lung carcinoma (A549) cell lines support the growth of these genotypes of HEV (Kamar et al. 2012). An HEV3 strain from a chronically infected patient has been adapted to grow in HepG2/C3A hepatoma cells (Kamar et al. 2012).

3.4. Infection and Disease

The HEV produces Acute Jaundice Syndrome (AJS) that does not obviously differ from the disease caused by HAV. Asymptomatic infections exceed the number of clinical illnesses. Following a 2–8 week incubation period, symptoms of viremia, nausea, fever, abdominal pain, arthralgia, dark urine and general malaise may develop in addition to the classic appearance of jaundice. In developing countries, where HEV1 and HEV2 predominate, case fatality rates during epidemics have been reported between 0.2 and 4 % for non-pregnant persons, with higher mortality in children under 2 years of age. Mortality rates for pregnant females are as high as 10 % and are more likely to occur in the third trimester.

In developed countries where HEV3 and HEV4 predominate, the majority of infections are asymptomatic. Clinical (icteric) infections are generally mild and self-limiting, lasting 4–6 weeks unless an underlying chronic liver disease is present. Excess fatalities among pregnant women have not been reported for HEV3 or HEV4 (Dalton et al. 2013). Symptomatic infection is reported to be 3 times more common in middle-aged and elderly men than women, even though seroprevalence rates in men and women are similar. Reasons for this discrepancy are not clear, but are probably linked to excessive alcohol consumption among a cohort of this population. Chronic infection occurs primarily among immunosuppressed patients, particularly solid organ transplant recipients. It is estimated that HEV prevalence among transplant patients is 1.0–2.3 % and although infection is usually asymptomatic, approximately 10 % of exposed patients develop cirrhosis within 2 years. Infrequently, neurological disorders, encephalitis and muscular impairments (including Guillain-Barré syndrome) have resulted from acute and chronic infection with HEV3 and possibly also HEV1. Viremia and virus excretion in feces through bile begins 2 weeks before the peak elevation of liver enzymes (and symptoms, if present) and continues until the enzyme levels return to normal (up to 2–3 weeks after symptoms appear). Diagnosis is generally by detection of IgM and IgG in patients' sera or by molecular detection of the virus in feces or sera. A recombinant vaccine was licensed in China in 2012 but has not yet received WHO recommendation (Dalton et al. 2013).

3.5. Foodborne Disease

Water contaminated with human and/or swine waste can harbour HEV allowing it to enter the food chain if used for shellfish production or irrigation. Similarly, fecal contamination of run-off waters from pig farms or from lands spread with untreated pig manure could contaminate irrigation and surface waters with subsequent HEV contamination of fruits, vegetables and shellfish. The virus has been detected in raw sewage, river water, shellfish and seawater in Europe, Japan, Southeast Asia, New Zealand, UK and the U.S. (Clemente-Casares et al. 2003; Crossan et al. 2012; Ishida et al. 2012; Jothikumar et al. 1993; Masclaux et al. 2013; Pina et al. 2000; Pina et al. 1998; Williamson et al. 2011). Therefore, consumption of raw or undercooked shellfish is a risk factor for HEV infection and has been implicated as the source of sporadic cases occurring in Europe and Southeast Asia (Cacopardo et al. 1997; Koizumi et al. 2004). In addition, HEV has been detected on strawberries irrigated with river water under experimental conditions (Brassard et al. 2012), providing evidence for HEV contamination through irrigation water. In a study where enteric viruses were traced through a food production chain, 1 of 38 samples of frozen raspberry tested positive for HEV, but the source (water or human/animal contact) of viral contamination could not be definitively identified (Maunula et al. 2013).

3.6. Zoonotic Transmission

The HEV has now been established as a zoonotic pathogen. Swine are the primary reservoir for HEV3 and HEV4 strains but infections have been reported in a wide range of wild and domestic animals. Human HEV3 and HEV4 strains can easily infect pigs under experimental conditions indicating a zoonotic origin for these viruses, a property not shared by human HEV1 and HEV2 strains (Meng 2011). The virus replicates in the liver and intestine and is shed in the feces and bile of swine for 3–5 weeks following infection (Halbur et al. 2001). It is highly contagious and therefore can spread among herds of domestic pigs very efficiently (Dalton et al. 2013). Swine veterinarians and handlers are significantly more likely to be seropositive for anti-HEV antibodies (Meng 2011), a finding consistent with HEV transmission through contact or environmental exposure.

Animal meat can also be contaminated with HEV via infection of the liver or by contact with infected feces during animal dressing or meat processing. A recent study conducted in the United States showed that 11 % of pig livers tested positive for HEV RNA and the virus was infective (Feagins et al. 2007). In Japan, 2 % of commercial pig livers were HEV positive and the strains were closely related to those detected in HEV-infected humans (Yazaki et al. 2003). Consumption of raw or undercooked pig and wild boar livers was a risk factor for several sporadic HEV illnesses recently reported in southeast Asia and Europe (Colson et al. 2010; Li et al. 2005; Masuda et al. 2005; Matsubayashi et al. 2008; Matsuda et al. 2003). Zoonotic transmission of HEV was also reported after consumption of raw deer meat by two Japanese families (Takahashi et al. 2004; Tei et al. 2003). Tei et al. (2004) investigated the risks associated with consumption of uncooked deer meat in a case control study and found that, in the area studied, eating uncooked deer meat was a risk factor (Tei et al. 2004).

4. NOROVIRUS

4.1. Distribution and Transmission

Noroviruses, previously known as small round structured viruses (SRSVs) and Norwalk-like viruses (NLVs), are now the most widely recognized viral agents associated with food and waterborne outbreaks of non-bacterial gastroenteritis and probably the most common cause of foodborne disease worldwide. The prototype norovirus, the Norwalk virus, was first discovered by Kapikian et al. (1972) following an outbreak of gastroenteritis in a school in Norwalk, Ohio (Kapikian et al. 1972). Immune electron microscopy was used to examine feces from volunteers who consumed fecal filtrates from infected cases (Dolin et al. 1971; Kapikian et al. 1972). At that time, most cases of gastroenteritis that could not be attributed to a bacterial agent were termed as acute nonbacterial gastroenteritis of unknown etiology. The discovery of

Norwalk virus provided the first evidence of a viral etiology for human diarrheal disease. Despite this discovery, noroviruses remained largely unrecognized until about 20 years ago because their detection was technically difficult and because the illness was generally thought to be mild and short-lived and so was frequently not reported to public health authorities.

Noroviruses are primarily transmitted by direct person-to-person spread, via the fecal-oral route, or by aerosolized vomit. Indirect transmission can also occur after consumption of fecally contaminated food or water, or after contact with contaminated surfaces, including high-touch surfaces (i.e. door handles, faucets, utensils) or those impacted by an ill person's vomitus. Outbreaks commonly occur in closed community situations such as rest homes, schools, camps, hospitals, resorts and cruise ships where many high-touch surfaces and food and water sources are shared. Since norovirus infections are often not notifiable, the global burden of disease is not known and is generally grossly under-reported particularly in developing countries. However, some of the disease burden is recorded in many developed countries through the notification of gastroenteritis outbreaks to the public health disease surveillance systems. Human noroviruses cause ~90 % of all outbreaks of non-bacterial gastroenteritis and ~50 % of all-cause outbreaks worldwide (Patel et al. 2009). The National Outbreak Reporting system (NORs), launched in 2009 by the CDC in the United States, determined that norovirus was responsible or suspected in 89 % of all outbreaks of gastroenteritis with a known etiology (Wikswo and Hall 2012).

Noroviruses are also responsible for approximately 58 % of foodborne disease in the US, including 5.5 million cases, 26 % of hospitalizations and 11 % of deaths related to foodborne disease each year (Scallan et al. 2011). In New Zealand, norovirus is the most frequently reported agent for gastroenteritis outbreaks. Between 2001 and 2007, 809 reported outbreaks of norovirus infection involving a total of 18,508 cases, were reported in annual surveillance reports (Greening et al. 2009). Norovirus was also confirmed as the cause of 1206 gastroenteritis outbreaks between 2002 and 2009, of which 64.6 % occurred in healthcare settings and 16.6 % were associated with catered settings and shellfish consumption (Greening et al. 2012). Recent estimates published by the Food-borne Viruses in Europe Network indicated that 21 % of all norovirus outbreaks are estimated to be due to foodborne transmission (Verhoef et al. 2010). Although noroviruses contribute significantly to foodborne disease, the primary means of norovirus transmission appears to be person-to-person worldwide.

4.2. Taxonomy and Morphology

There are five genera in the family *Caliciviridae*: *Norovirus* and *Sapovirus* are human pathogens while *Lagovirus*, *Vesivirus*, and the newly discovered *Nebovirus* infect animals and are not known to be pathogenic for humans. Two additional genera, *Recovirus* and *Valovirus*, have recently been proposed. The former includes the Tulane virus which was isolated from rhesus macaques

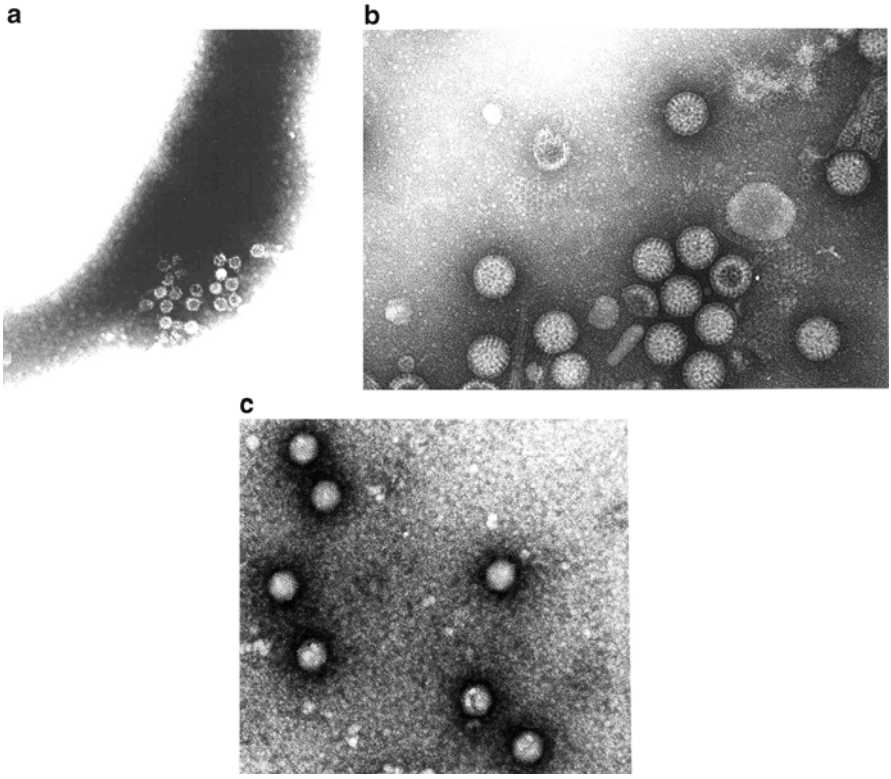

Figure 2.1. Electron micrographs of human enteric viruses. Negative staining. Bar represents 100 nm. (**a**) Baculovirus-expressed recombinant Norwalk virus-like particles (VLPs); (**b**) rotavirus; (**c**) adenovirus.

(Farkas et al. 2008) and the latter, St-Valérien-like viruses, which have been isolated from swine (L'Homme et al. 2009). The 'Norwalk-like viruses' and 'Sapporo-like viruses' were renamed as *Norovirus* and *Sapovirus* in August 2002 by the ICTV (Van Regenmortel 2000). The noroviruses do not show the characteristic cup-shaped morphology of caliciviruses but instead show a "fuzzy" or ragged appearance by direct electron microscopy, which is why they were classified as a distinct group until 1995 (Fig. 2.1a).

The noroviruses are 28–35 nm non-enveloped, linear, positive sense, single-stranded RNA viruses with a genome of approximately 7.4–7.7 kb and icosohedral capsid symmetry (Table 2.1). The buoyant density in cesium chloride gradient is 1.36–1.41 g/ml. The genome is composed of three open reading frames (ORF), which code for the non-structural proteins, including the RNA polymerase in ORF1, the major capsid protein (VP1) in ORF2 and a minor structural protein (VP2) in ORF3. Currently, six norovirus genogroups (GI, GII, GIII, GIV, GV and GVI) have been identified. The majority of human infections are caused by genogroup I and II (GI & GII) strains but infections

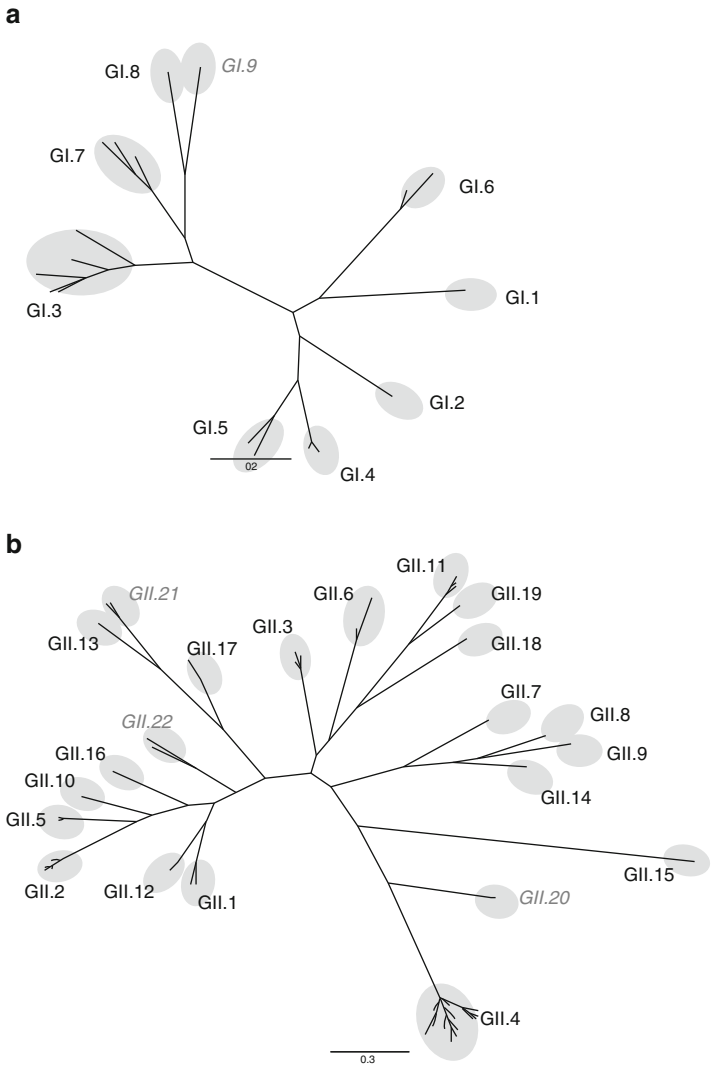

Figure 2.2. Phylogenetic trees of complete capsid amino acid sequences of norovirus GI (**a**) and GII (**b**), showing all genotype clusters as defined in Fields Virology (Green 2007) and one new GI cluster (GI.9) and three new GII clusters (GII.20-22). Figure taken from Kroneman et al. (2013).

with GIV strains are sometimes reported. Genogroups I and II contain 9 (GI.1-9) and 22 genotypes (GII.1-22), respectively (Kroneman et al. 2013) (Fig. 2.2). Genogroup II includes three genotypes (GII.11, GII.18 and GII.19) that cause asymptomatic infection in swine. Two genotypes of genogroup III (GIII.1-2) are known to cause diarrhea in bovine calves. Genogroup IV noro-

viruses have been detected in humans (GIV.1), dogs, lions and cats (animal strains belong to GIV.2) (Martella et al. 2007; Martella et al. 2008b; Pinto et al. 2012). Genogroup V noroviruses are highly prevalent in laboratory mice but are not known to infect other animals (Wobus et al. 2006). The most recently established genogroup VI viruses infect canine species (Green 2013). An international norovirus working group established in 2008 recently published new parameters for norovirus genotyping and proposed a standard nomenclature for reporting purposes (Kroneman et al. 2013). In addition, an online typing tool (http://www.rivm.nl/mpf/norovirus/typingtool) is now available for public use.

4.3. Growth and Biological Properties

Most information on the biology and properties of noroviruses has been obtained through studies in human volunteers and laboratory animals with culturable animal caliciviruses. Human volunteer studies primarily conducted in the 1970s (Dolin et al. 1972; Dolin et al. 1971; Green 2007) and more recently in the 2000s (Atmar et al. 2011; Atmar et al. 2008; Hutson et al. 2002; Lindesmith et al. 2003) have almost exclusively been conducted with the prototype GI.1 Norwalk virus, although two studies were performed with GII.2 and GII.4 norovirus (Frenck et al. 2012; Lindesmith et al. 2005).

Despite numerous attempts of *in vitro* culture in traditional cell lines, primary cultures, or three-dimensional cultures grown to simulate intestinal tissues, human noroviruses remain recalcitrant to routine culture in the laboratory (Duizer et al. 2004; Lay et al. 2010; Papafragkou et al. 2013). However, recently Jones et al. (2014) reported low-level HuNoV infection in cultured B-cells which could be enhanced by co-inoculation with bacteria expressing specific carbohydrate molecules known as histo-blood group antigens (Jones et al. 2014). While an exciting breakthrough, further optimization is needed to increase its robustness and applicability in food virology laboratories. Permissive cell lines have been identified that are capable of producing genome-packaged, intact virus particles after transfection of virus-derived genomic RNA, but viruses were not capable of infecting new cells, even after over-expression of the putative cellular receptors for HuNoVs (Guix et al. 2007). Replication of HuNoVs in laboratory animals has been described in gnotobiotic pigs and calves and non-human primates (Bok et al. 2011; Tan and Jiang 2010), but such animals are not well suited for routine laboratory studies. Evidence of HuNoV replication in the systemic tissue of immunocompromised laboratory mice was recently reported (Taube et al. 2013), suggesting the possibility for a mouse model for human norovirus gastroenteritis in the near future. Several animal caliciviruses, including a murine norovirus (MNV), can be grown in routine laboratory cultures. Animal models in gnotobiotic pigs and calves have also been described (Tan and Jiang 2010). Much information regarding the basic virology and pathogenesis of noroviruses can be inferred from these models, but many questions remain unanswered.

The development of assays such as RT-PCR has facilitated the detection and identification of these viruses, and consequently the role of noroviruses in gastroenteritis outbreaks has been clarified. Noroviruses show great genetic diversity, which has complicated their identification by molecular assays. A region at the junction and overlap of ORF1 and ORF2 is highly conserved among noroviruses of the same genogroup. Therefore, broadly reactive primers and probes targeting this region have been developed for clinical diagnosis and environmental detection using both conventional and real time RT-PCR platforms (CDC 2011).

The NoV capsid proteins (VP1 or VP1/VP2) can be expressed *in vitro* and self-assemble into virus-like particles (VLPs) which are antigenically similar to native viruses. The non-infectious VLPs are highly immunogenic, making them suitable for antiserum and vaccine production (Atmar et al. 2011; Estes et al. 2000). However, many first- and second-generation commercial ELISA assays were reported to have limited sensitivity and specificity (Burton-MacLeod et al. 2004; Richards et al. 2003). A third-generation antigen detection assay, RidaScreen III, has proven to be a useful screening tool for rapid diagnosis during norovirus outbreaks, yet is still not recommended for clinical diagnosis or screening of sporadic illnesses (Morillo et al. 2011).

Since norovirus is non-culturable, its infectivity can only be assessed by human challenge or estimated using surrogate viruses. Studies using human volunteers showed that norovirus retains infectivity when heated to 60 °C for 30 min and therefore is not inactivated by pasteurization treatment although studies with infectious norovirus surrogates indicate pasteurization temperatures are sufficient for inactivation (Bozkurt et al. 2013; Cannon et al. 2006). The virus also retains infectivity following exposure to pH 2.7 for 3 h at room temperature (Dolin et al. 1972; Dolin et al. 1971; Green 2007). Further evidence of its resistance to low pH was shown when norovirus was exposed to heat treatment and subsequent marination at pH 3.75 in mussels for 1 month; no decrease in norovirus titer was observed by real-time RT-PCR (Hewitt and Greening 2004). There are anecdotal reports of people developing gastroenteritis after eating pickled shellfish. Norovirus, like other enteric viruses, remains infectious under refrigeration and freezing conditions, appears to survive well in the environment, and is resistant to drying. This was demonstrated when carpet layers became ill after lifting carpet that had become contaminated 12 days earlier in a retirement home outbreak (Chadwick et al. 2000). In a recent study, HuNoV remained infectious in groundwater for at least 61 days (last collection point for human challenge study) and viral RNA could be detected 3 years after inoculation (Seitz et al. 2011).

Fecal pollution from sewage discharges, septic tank leachates and boat discharges has caused contamination of shellfish beds, recreational water, irrigation water and drinking water. It is probable that noroviruses persist in these environments for extended periods (weeks or months). In infected live oysters, noroviruses were still detectable after 4–6 weeks in natural growing conditions (Greening et al. 2003). Fecal material associated with these viruses and their

propensity to aggregate causes them to be more moderately resistant to chemical disinfectants, including free chlorine levels used in drinking water distribution systems. Noroviruses also retain at least partial infectivity after exposure to 20 % ether at 4 °C for 18 h (Dolin et al. 1972; Green 2007). It should be noted, however, that virus titer could not accurately be determined in the early human challenge experiments reviewed in Green (2007). If starting virus titers are high (i.e. ≥8 log per ml), incomplete virus inactivation may be observed even if a disinfectant produces 3–4 $\log_{10}$ reduction in the titers of infectious virus.

4.4. Infection and Disease

Noroviruses are extremely infectious; the 50 % infectious dose of Norwalk (GI.1 strain) virus in humans was recently calculated to be as low as 18 (Teunis et al. 2008) and as high as 2800 virus particles (Atmar et al. 2014). Very high numbers (10^{10}–10^{12} per g) of noroviruses can be shed in feces of symptomatic and even asymptomatic persons (Atmar et al. 2008). Symptoms normally include vomiting, diarrhea, nausea, abdominal pain and general malaise, but vomiting can be present without diarrhea and vice versa. Viruses are excreted in vomit and feces during illness and can continue to be shed in decreasing numbers for up to 28 days or longer in stools (Atmar et al. 2008). Symptoms normally develop within 12–48 h of exposure and resolve within 24–60 h. Dehydration is a common complication that can particularly affect the young and elderly, necessitating rehydration therapy. There is no evidence of any long-term sequelae following norovirus infection.

In the absence of reliable laboratory tests for norovirus, Kaplan et al. (1982) developed epidemiological and clinical criteria for the diagnosis of noroviral gastroenteritis outbreaks (Kaplan et al. 1982). These widely used criteria are: stools negative for bacterial pathogens, a mean or median duration of illness of 12–60 h, vomiting in ≥50 % of cases and a mean or median incubation period of 24–48 h. The mechanism of immunity to norovirus infection is not clear. Infection normally stimulates production of both gut and serum antibody and although immunity to the infecting norovirus strain may develop, it is generally short-lived, is strain-specific and does not confer protection against future infection. Re-infection with a different strain can occur soon after the initial infection. Thus, given the genetic variability of noroviruses, people are likely to be re-infected many times during their lifetimes.

Several reports over the last 10 years have revealed an association between histo-blood group antigen (HBGA) expression on a person's gut mucosal cells and a susceptibility to norovirus infection (Atmar et al. 2011; Hutson et al. 2002; Lindesmith et al. 2003; Tan and Jiang 2010). Persons deemed to be "secretors" are capable of expressing HBGAs on their gastrointestinal cells and secretions, including saliva. Noroviruses are capable of binding to HBGAs and saliva from secretors and the role of HBGAs as receptors or co-receptors for virus attachment and entry into cells has been speculated. Evidence for this was derived from human challenge studies with Norwalk virus (GI.1 proto-

type) where "non-secretors", individuals that do not express HBGAs on their GI mucosa, were resistant to symptomatic infection (Atmar et al. 2011; Hutson et al. 2002; Lindesmith et al. 2003; Tan and Jiang 2010). There also appears to be an association with HBGA expression and susceptibility to infection with GII.4 noroviruses (Frenck et al. 2012; Tan and Jiang 2010); although, this association is not as well defined as it is for Norwalk virus. There is also evidence that susceptibility to infection by other norovirus genotypes may not depend on the secretor status (Lindesmith et al. 2005). Considering the genetic diversity of noroviruses, this unfortunately means that there is likely a genotype capable of infecting almost everyone, regardless of their HBGA expression.

Projectile vomiting is a characteristic symptom that can contribute to secondary spread through droplet infection, where droplets containing virus may contaminate surfaces or be swallowed. Evidence that norovirus transmission occurs through aerosolization of vomit was clearly demonstrated at a UK hotel. During a meal, a guest vomited at the table and norovirus infection spread in a radial pattern through the restaurant, progressively decreasing from 91 % attack rate among those seated at the same table to an attack rate of 25 % in those patrons who were seated the furthest distance away from the guest who vomited (Marks et al. 2000). Norovirus infection characteristically has an attack rate of 50–70 % or even higher in some situations. High attack rate, low infectious dose, prolonged virus excretion, short-term immunity and, environmental stability of NoVs contribute to the epidemic nature of noroviral gastroenteritis.

Norovirus infection was historically termed 'winter vomiting disease' because outbreaks occurred most frequently in the winter months, especially in rest homes and institutions. This seasonality is still apparent, but NoV outbreaks are also reported throughout the year. Viruses belonging to GII.4 are by far the most frequently reported in outbreaks and sporadic illnesses worldwide. This topic will be discussed in greater detail in Chap. 4 of this book.

4.5. Foodborne Disease

Noroviruses are the main cause of foodborne viral gastroenteritis worldwide with foodborne transmission accounting for a large proportion of NoV outbreaks in many countries. Foodborne norovirus outbreaks resulting from preharvest contamination of foods such as shellfish and post-harvest contamination through food handling have been reported worldwide. Among these are several outbreaks resulting from consumption of norovirus-contaminated shellfish (Dowell et al. 1995; Christensen et al. 1998; Berg et al. 2000; Simmons et al. 2001; Bellou et al. 2013), bakery products (Kuritsky et al. 1984; de Wit et al. 2007), delicatessen and ready to eat meats (Schwab et al. 2000; Malek et al. 2009), sandwiches (Parashar et al. 1998; Daniels et al. 2000; CDC 2006), raspberries (Ponka et al. 1999; Hjertqvist et al. 2006; Maunula et al. 2009; Sarvikivi et al. 2012), and water and ice (Beller et al. 1997; Brugha et al. 1999; Beuret et al. 2002). Pre-symptomatic infection in food handlers has also been shown to cause outbreaks of foodborne norovirus infection (Lo et al. 1994; Gaulin et al. 1999; Thornley et al. 2013).

Among the 2922 confirmed or suspected outbreaks of foodborne norovirus illness reported to CDC from 2001 to 2008, the food vehicle could not be determined for 56 % of the outbreaks (Hall et al. 2012). Of the 886 outbreaks where the factors leading to food contamination were indicated, 82 % of outbreaks involved food handler contact and 13 % indicated contamination of the raw product (Hall et al. 2012). Determination of the original source of virus is often problematic because several modes of transmission frequently operate during norovirus gastroenteritis outbreaks. Although the initial transmission route may be through consumption of contaminated foods, secondary transmission via direct contamination of the environment or person-to-person contact also often occurs. This results in wide dissemination where infection quickly spreads through institutions, schools, camps and resorts and cruise ships leading to large-scale epidemics, often with over 50 % attack rates.

The use of DNA sequencing techniques for genotyping of noroviruses has greatly assisted the epidemiological investigation of gastroenteritis outbreaks. The comparison of norovirus sequences from fecal specimens and contaminated foods, such as oysters, can often indicate if it is a common source outbreak or if individual cases are somehow related, particularly when a strain is involved that is not common in the community. In 1993, 23 gastroenteritis outbreaks across six states in the United States were shown to be related to consumption of oysters harvested from a single area and contaminated with the same norovirus strain (Dowell et al. 1995). Validating the source of outbreaks caused by GII.4 strains is often more difficult because these strains can be highly prevalent in the community, particularly during pandemic years. Sequencing either the full capsid-encoding ORF2 or minimally the hypervariable region of ORF2 is needed for molecular epidemiologic investigations of these strains.

4.6. Zoonotic Transmission

To date, zoonotic transmission of noroviruses remains speculative. Despite the presence of GII norovirus strains in swine populations (0.2–25 % detection rate) globally, there is no evidence of human infection by these strains (Mathijs et al. 2012). Similarly, GIII bovine noroviruses have been detected in the stools of diarrheic calves with a 1.6–72 % detection rate (Mathijs et al. 2012). Despite serologic evidence of large animal veterinarians' exposure to bovine noroviruses (Widdowson et al. 2005), no illnesses have been reported. GIV.1 noroviruses appear to infect only humans and animal strains of GIV norovirus have not been detected in humans. Recombination between strains within the same host species has been reported for human, bovine, canine and mouse noroviruses; however, recombination between strains of different host species has not been confirmed. In the laboratory, HuNoV infection in gnotobiotic pigs and calves (Cheetham et al. 2006; Souza et al. 2008; Takanashi et al. 2011) and non-human primates (Bok et al. 2011; Rockx et al. 2005) has been extensively studied as models for viral pathogenesis, immunology and response to vaccination (Tan and Jiang 2010). Only two reports to date

provide evidence of animals as reservoirs for human noroviruses. In one study, human norovirus-like sequences were detected in the feces of livestock (cows and pigs) (Mattison et al. 2007). Human norovirus RNA was also detected in the feces of pet dogs from households where symptoms of gastroenteritis were reported, suggesting a potential role for dogs in HuNoV transmission (Summa et al. 2012). However, based on current evidence it seems more likely that dogs are the recipients of spill-over from the human population rather than being a reservoir.

5. SAPOVIRUS

5.1. Distribution and Transmission

The sapoviruses, formerly described as the "Sapporo-like viruses" or SLVs, also belong to the *Caliciviridae* family and cause gastroenteritis among both children and adults. Sapoviruses were first identified in 1977 after a gastroenteritis outbreak in a children's home in Sapporo, Japan. Sapoviruses were once thought to be primarily associated with pediatric diarrhea in infants since infections among adults were less frequently reported and were less severe than those caused by HuNoVs (Hansman et al. 2007a). Diagnostic testing for sapovirus is, therefore, infrequently performed during routine outbreak investigations or after emergency room visits. However, outbreaks of sapovirus gastroenteritis among adults and elderly persons are increasingly reported worldwide (Pang et al. 2009; Svraka et al. 2010). In addition, the emergence of specific sapovirus strains (GI.2 and GIV) has correlated with its increased occurrence (Gallimore et al. 2006; Khamrin et al. 2007; Lee et al. 2012; Svraka et al. 2010). Sapovirus infections occur throughout the year, but the frequency is higher during the winter and spring months. Person-to-person transmission is believed to be the most common route, although several foodborne outbreaks have been reported (Hansman et al. 2007b; Iizuka et al. 2010; Kobayashi et al. 2012; Nakagawa-Okamoto et al. 2009; Noel et al. 1997; Ueki et al. 2010; Yamashita et al. 2010).

5.2. Taxonomy and Morphology

Sapoviruses show a Star-of-David structure under EM, with distinct cup-shaped indentations typical of caliciviruses on the surface and/or ten spikes on the outline of the virions. The sapoviruses are 30–38 nm, non-enveloped, positive sense, single-stranded RNA viruses with a genome of approximately 7.1–7.7 kb and icosohedral capsid symmetry (Table 2.1) (Oka et al. 2015). 'Sapporo-like viruses' were renamed as *Sapovirus* in 2002 by the ICTV (Van Regenmortel 2000). There are currently five sapovirus genogroups (GI-GV), but eight additional genogroups (GVI-GXIV) have been proposed (Oka et al. 2015). GI, GII, GIV and GV viruses infect humans. Based on the nucleic acid sequence of the major capsid protein (VP1), human sapoviruses are

further divided into at least seven GI and GII genotypes, one GIV genotype and two genotypes of GV (Oka et al. 2012; Oka et al. 2015). GV genotypes also have been detected in pigs and sea lions (Oka et al. 2015). Of the additional proposed genogroups, three infect swine (GVI, GVII and GVIII) (Scheuer et al. 2013). The other proposed genogroups of sapovirus have been detected in swine, mink, dogs, and bats (Scheuer et al. 2013).

5.3. Growth and Biological Properties

A majority of sapovirus strains are not capable of growth in cell culture. Detection and identification is generally by molecular methods, most commonly RT-PCR (Green 2007; Hansman et al. 2007a). However, the GIII-Cowden strain of porcine sapovirus (historically referred to as porcine enteric calicivirus or PEC) has been adapted to grow in a continuous swine kidney cell line (LLC-PK). Its culture depends on supplementation of media with glycochenodeoxycholic acid (GCDCA), a bile acid (Chang 2004). This strain has recently been proposed as a surrogate for HuNoVs to be used in disinfection and survival studies (Wang et al. 2012).

5.4. Infection and Disease

Although previously thought to cause primarily diarrhea, a high percentage (37–89 %) of vomiting among cases have recently been reported (Chhabra et al. 2013; Iizuka et al. 2010; Kobayashi et al. 2012; Lee et al. 2012; Yamashita et al. 2010; Yoshida et al. 2009). In fact, the clinical symptoms, virus shedding, and epidemiological features (incubation period, duration of illness) of epidemic sapovirus are nearly identical to those of noroviruses. It is hypothesized that using the Kaplan criteria alone for diagnosis of outbreaks may result in an overestimation of norovirus and an underestimation of sapovirus (Lee et al. 2012). Severe gastrointestinal illnesses and even deaths due to sapovirus do occur, but are infrequently reported. In a recent study, norovirus- and rotavirus- negative clinical specimens collected from children seeking medical attention for acute gastroenteritis were screened for less common agents of viral gastroenteritis; 5.4 % were positive for sapoviruses (Chhabra et al. 2013). However, determining sapovirus as the causative agent of gastroenteritis is sometimes difficult, particularly for sporadic cases, due to the common presence of other gastroenteritis-causing viruses in fecal specimens and high rates of asymptomatic carriage. In one study, 4.2 % of the healthy control specimens tested positive for sapovirus and mixed infections with rotavirus, norovirus, astrovirus and adenovirus were reported (Chhabra et al. 2013). High levels of sapovirus shedding (10^6–10^{11} per g) in stools from both symptomatic and asymptomatic persons have been reported (Yoshida et al. 2009). While the infectious dose is not known, it is thought to be low and similar to that for noroviruses.

5.5. Foodborne Disease

Food handler contamination of RTE foods and incidences of contaminated shellfish harvesting waters are increasingly being reported. One of the first food-related outbreaks of sapovirus gastroenteritis occurred among adults at a school in Parkville, Maryland, in 1997 (Noel et al. 1997). In Japan, there have been several reports of sapovirus outbreaks due to the consumption of oysters (Nakagawa-Okamoto et al. 2009; Ueki et al. 2010) and clams (Hansman et al. 2007b; Iizuka et al. 2010). A large food handler-associated outbreak was reported among 109 wedding guests served boxed lunches (Yamashita et al. 2010). In January 2010, the largest outbreak of sapovirus ever to be reported in Japan (655 persons; 17 % of persons served) was also caused by boxed lunches contaminated by food handlers (Kobayashi et al. 2012).

5.6. Zoonotic Transmission

There are no reports of zoonotic transmission of sapoviruses to date. However, the potential exists as intra- and inter-genogroup recombination has been reported (Hansman et al. 2007a; Wang et al. 2005). Porcine sapoviruses co-concentrated with other human enteric viruses have been detected in raw US oysters, revealing a potential vector for mixed infections and recombination (Costantini et al. 2006). In addition, closely related GVIII sapovirus strains have been detected in humans and swine indicating possible transmission of GVIII strains from humans to pigs or vice versa (Martella et al. 2008a; Scheuer et al. 2013).

6. ROTAVIRUS

6.1. Distribution and Transmission

Acute gastroenteritis caused by rotaviruses is a common disease of infants and young children worldwide, which can lead to severe illness and deaths especially in locations where medical services are not readily accessible. Although the disease occurs in all age groups, it is generally considered to be a mild infection in adults; hence, the true extent of adult infections is not known. Since the licensing of two live, oral, second-generation rotavirus vaccines in 2006, there has been a marked decline in the number of diarrheal illnesses caused by rotaviruses in young children, particularly in the developed world. In the United States, where rotaviruses were estimated to cause about 3 million infections, 70,000 hospitalizations and nearly 100 deaths annually, the impact of routine childhood vaccination on the reduction of rotavirus infections is already apparent (Glass et al. 2011; Malek et al. 2006; Parashar et al. 2006a).

Vaccination of U.S. children with RV5 RotaTeq® (Merck and Company, Inc.) has decreased the number of hospitalizations due to rotaviruses by up to

89 % (Cortes et al. 2011). In addition, the annual number of diarrheal illnesses due to rotavirus has also been in steady decline (Glass et al. 2011; Tate et al. 2011). The pentavalent RV5 and monovalent RV1 Rotarix® (GlaxoSmithKline) vaccines have been recommended by WHO for all regions of the world since 2009 (WHO 2009) and have been licensed in more than 100 countries (Glass et al. 2011). In many regions of the world, rotaviruses are still a major cause of severe acute gastroenteritis, causing more than 100 million cases worldwide of childhood diarrhea, 2.4 million hospitalizations and 500,000 deaths in children under the age of 5 years in developing countries at the time of vaccine release (Greenberg and Estes 2009; Malek et al. 2006; Parashar et al. 2006a; Parashar et al. 2006b). Although it is clear that second-generation vaccines have had a significant impact on several middle- and high-income countries, vaccine efficacy has generally not been as high as in low-income countries, for reasons not entirely known (Glass et al. 2011; Jiang et al. 2010; Yen et al. 2011). As the poorest regions of the world suffer the greatest numbers of severe illnesses and deaths due to rotavirus, there is a critical need to understand the underlying causes of this disparity and improve vaccination efficacy in these populations.

Rotaviruses are transmitted by the fecal-oral route and cause disease in both humans and animals, especially domestic animals, with subsequent serious economic loss. Although the animal and human strains are usually distinct, some human strains are closely related to animal strains and cross-species infections do occur (Sattar and Tetro 2001). Rotaviruses can be excreted in human stool at concentrations of up to 10^{12} virus particles per g of feces, making them highly transmissible through direct person-to-person contact and by contaminated fomites (Bishop 1996). Transmission of rotavirus via aerosolized droplets following a vomiting event has not been confirmed. As few as ten virus particles appear to be sufficient for causing infection in humans (Graham et al. 1987). Infection is generally not recognized as being foodborne but outbreaks associated with food and water have been reported from a number of countries (Sattar and Tetro 2001).

6.2. Taxonomy and Morphology

Rotaviruses are classified in the genus *Rotavirus* in the family *Reoviridae*, a large family comprised of two virus subfamilies, *Spinareovirinae* and *Sedoreovirinae*, which include nine and six genera, respectively (Carstens 2010). Rotaviruses belong to the *Sedoreovirinae* subfamily. Electron micrographs of rotaviruses show a characteristic wheel-like appearance, hence the name ‘rotavirus’, derived from the Latin meaning ‘wheel’ (Fig. 2.1b). These viruses are distinct in that they have a complex, segmented genome that undergoes reassortment during replication. There are eight groups (also termed species) within the Rotavirus genus, designated A-H (Desselberger 2014). Group A rotaviruses are most commonly associated with human disease; infection with Groups B and C is rarely reported. Group A rotaviruses

are also common agents of disease in cattle and several other domestic animal species (equine, porcine, ovine, caprine, canine, feline (rare), and poultry (rare)) (Dhama et al. 2009). In addition, Groups C and E rotaviruses infect swine; Groups D, F and G affect poultry; and Group B viruses have been detected in young cattle, swine and sheep, although rarely (Dhama et al. 2009). Initially isolated from humans but unassigned to a rotavirus group until recently, Group H rotaviruses have been reported to infect both humans and piglets (Molinari et al. 2014).

Rotaviruses are 60–80 nm, non-enveloped viruses with icosohedral capsid symmetry and a linear, segmented, double stranded RNA genome. The 16–27 kb genome is enclosed in a triple layered capsid composed of a double protein shell and an inner core. The 11 segments of RNA code for 11 proteins (six structural and five non-structural). Their classification has been traditionally based on the antigenic properties of two structural proteins, VP7 (G-type glycoprotein) and VP4 (P-type protease-sensitive protein), which comprise the shell and spike proteins of the outer capsid, respectively, and are important in virus infectivity and immunogenicity. The proteins have been used to define P and G serotypes of rotaviruses (Estes and Kapikian 2007). However, serotyping is time consuming and immunological reagents are not always readily available. While there is good correlation between G serotypes and VP7 genotypes, P serotype and VP4 genotyping results are not always in agreement (Greenberg and Estes 2009), so classification is now mainly based on sequencing of these genes or a double nomenclature for P types, based on genotype and serotype information (Desselberger 2014). Genotyping by sequencing is based on identities between sequences of cognate gene segments. There are at least 27 G genotypes and 37 P genotypes within the Rotavirus A (RVA) species (Desselberger 2014). Predominant strains circulating globally include six G types (G1-4, G9, and G12) and two P types (P[4] and P[8]). The six G- and P-type combinations accounting for 80–90 % of all rotavirus infections in North America, Europe and Australia include, G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] and G12P[8] (Desselberger 2014). In Africa, G5, G6 and G8 types predominate (Desselberger 2014). A Rotavirus Classification Working Group (RCWG) charged with developing a genome classification system for RVA was recently formed to address the lack of uniformity in the literature (Matthijnssens et al. 2008). In 2008, abbreviations for all 11 RNA segments were defined and percent cut-off values established for categorizing rotavirus strains using a common nomenclature (Matthijnssens et al. 2008). Furthermore, the RCWG proposed a standardized strain nomenclature system for reporting complete rotavirus genome sequences and for use in epidemiologic studies (RV group/species of origin/country of identification/common name/year of identification/G- and P-type). Since this time, 50 new genotypes have been reported and in several cases, a link between animal and human strains has been revealed (Greenberg and Estes 2009).

6.3. Growth and Biological Properties

Although many rotaviruses can be grown in cell cultures, they have proved difficult to cultivate *in vitro* and growth is restricted to a few cell lines derived mainly from monkey kidneys. Addition of trypsin to the culture medium is required to enhance viral growth in cell cultures. Rotaviruses do not show the same tolerance to extreme conditions as other enteric viruses although they are stable in the environment and can be stored for several months at 4 °C or even at 20 °C. They are resistant to drying and may survive on fomites and surfaces, but are labile under conditions of high relative humidity (approximately 80 %) (Estes and Kapikian 2007). Heating at 50 °C for 30 min reduces their infectivity by 99 % and infectivity is rapidly lost at pH <3.0 and >10.0. Repeated cycles of freeze-thaw can also destroy infectivity. The viruses are resistant to solvents such as ether and chloroform and to non-ionic detergents such as deoxycholate. Chelating agents such as EDTA disrupt the outer shell and inactivate rotaviruses. Treatment with disinfectants such as chlorine, phenol, formalin and 95 % ethanol is also effective against rotavirus (Estes and Kapikian 2007). Normal cooking temperatures are usually sufficient to inactivate rotaviruses. The viruses are found in water and sewage, are resistant to chlorine levels present in the drinking water distribution system and are persistent in the environment. Human rotavirus can survive for several weeks in river water at 4 and 20 °C.

6.4. Infection and Disease

The incubation period for rotavirus infection is 1–2 days. The characteristic symptoms of vomiting and watery diarrhea develop quickly and persist for 3–8 days, frequently accompanied by fever and abdominal pain. Dehydration is a key factor that contributes to the high infant death rate from rotavirus disease, especially in developing countries where rehydration therapy is often not readily available. Virus is shed in feces for 5–7 days. The main transmission route is fecal-oral. Since rotaviruses most often infect young children, the major route of transmission is believed to be person-to-person through care-givers and the general adult population. Rotaviruses, particularly group B, can also infect adults and have been occasionally associated with food and water outbreaks. In the early 1980s, a large waterborne epidemic affecting nearly 1 million persons was reported in China (Hung et al. 1984). Waterborne outbreaks have also been reported from Germany, Israel, Sweden, Russia, Turkey and the US (Ansari et al. 1991; Koroglu et al. 2011; Sattar and Tetro 2001). Rotavirus infections are more common during the winter months in countries with a temperate climate. In tropical regions, outbreaks can occur in cool, dry months as well as throughout the year especially when transmission is related to contaminated water supplies or in the absence of sewage treatment systems (Ansari et al. 1991; Cook et al. 1990).

6.5. Foodborne Disease

A case–control study examining risk factors outside the home implicated contact with a person suffering from gastrointestinal illness and poor hygienic kitchen practices as major contributors to the likelihood of contracting rotavirus infections (de Wit et al. 2003). Cross-contamination of foods during preparation in the home may be an important source of infection for children, particularly for weaning infants (Motarjemi et al. 1993). Eleven foodborne outbreaks consisting of 460 cases of rotaviral gastroenteritis were reported in New York between 1985 and 1990. Seven of these outbreaks were associated with food-service premises and the implicated foods included salad, cold foods, shepherd's pie, and water or ice (Sattar and Tetro 2001). In Japan, a foodborne rotavirus outbreak was reported among adults eating restaurant-prepared meals (Iizuka et al. 2000). College-age students in the United States became ill following consumption of tuna or chicken salad sandwiches prepared by food workers testing positive for the same P[4], G2 rotavirus strain that was detected in the stools of the students (CDC 2000).

Although no direct link between field-contaminated produce and outbreaks of rotavirus illness has been established as yet, several studies indicate that this route of transmission is possible. In Costa Rica, market lettuce was found to be contaminated with rotavirus and HAV at a time when there was a high incidence of rotaviral diarrhea in the community (Hernandez et al. 1997). Similarly, rotaviruses were detected on green onions and work surfaces within a packing house in Mexico (Felix-Valenzuela et al. 2012) and on strawberries irrigated with river water in Canada (Brassard et al. 2012). Sewage-impacted water has resulted in rotavirus contamination of shellfish in China (Ming et al. 2013) and was also the likely source of a gastroenteritis outbreak related to salad consumption on a cruise ship, where rotavirus, human norovirus and sapovirus were detected in the stools of ill persons (Gallimore et al. 2005).

6.6. Zoonotic Transmission

Rotaviruses are major pathogens of both humans and domestic animals. There is mounting evidence of zoonotic transmission between humans and animals, particularly when domestic animals are kept in close proximity to humans. Interspecies replication of rotaviruses occurs readily with many rotavirus strains, but is less commonly associated with disease. Exploitation of this fact was the basis for construction of live-attenuated rotavirus vaccine candidates. Due to the segmented nature of the viral genome, there is potential for genomic reassortment of human and animal rotaviruses. Close interspecies relationships between porcine-human and bovine-human strains have been identified by RNA-RNA hybridization and more recently by full genome sequencing. Several rotavirus strains that have caused illness in humans in Latin America, Asia, Europe and Africa have now been either confirmed or are highly suspected to have emerged after reassortment with animal

rotaviruses (Martella et al. 2010). Continued efforts to generate full-length genome sequences for human and animal rotavirus strains will thus be important for further elucidation of the zoonotic potential of rotaviruses.

7. ASTROVIRUS

7.1. Distribution and Transmission

Astroviruses are distributed worldwide and have been isolated from birds, cats, dogs, pigs, sheep, cows and man. The main feature of astrovirus infection in both humans and animals is a self-limiting gastroenteritis. Astroviruses are estimated to cause up to 20 % of all sporadic and 0.5–15 % of epidemic cases of non-bacterial gastroenteritis in humans, with most cases of infection being detected in young children under 2 years of age (De Benedictis et al. 2011). Although astroviruses cause a mild infection in adults, they have been associated with gastroenteritis in immunocompromised adults. Transmission is through the fecal-oral route via food, water and person-to-person contact, especially in nurseries, childcare centers and hospitals. Asymptomatic excretion is estimated to be approximately 10 % in most human populations but as high as 30 % prevalence rates have been reported in developing countries (De Benedictis et al. 2011; De Grazia et al. 2011; Guix et al. 2002). Co-infections with other enteric pathogens are common and have been reported to be between 17 and 65 % (De Benedictis et al. 2011).

7.2. Taxonomy and Morphology

The astroviruses, named for the star-like appearance they exhibit under electron microscope (EM), were first recognized in 1975 (Madeley and Cosgrove 1975). The virus belongs to the family *Astroviridae*, which includes the genera Mamastrovirus and Avastrovirus that infect mammals and birds, respectively. Astroviruses are 28–30 nm icosohedral, non-enveloped positive-sense single-stranded RNA viruses with a genome of about 6.8–7.8 kb and a buoyant density of 1.32 g/ml in potassium tartrate-glycerol gradient (Table 2.1). Since only 10 % of astroviruses exhibit the typical 5 or 6 pointed star-like morphology by direct EM, the efficiency of detection was restricted until the introduction of molecular detection methods and improved culture techniques. Eight human astrovirus species (HAstVs 1-8) have been identified based on serotyping but new species continue to emerge through molecular studies. Currently there are 33 genotypes in the Mamastrovirus genus and 11 genotypes in the Avastrovirus genus (Bosch et al. 2014). Mamastrovirus species are also detected in bovine, feline, porcine, canine, ovine, mink, rodent, bat and marine mammal hosts. Avastroviruses include turkey, chicken and duck astroviruses as well as the avian nephritis virus (De Benedictis et al. 2011).

7.3. Growth and Biological Properties

Wild-type astroviruses are fastidious to grow *in vitro*. Human, porcine, bovine and avian astroviruses have been adapted to grow in established cell lines, but trypsin is required in the growth medium to boost infectivity for all astroviruses except chicken astroviruses. Propagation of reference strains and wild-type astroviruses from stool suspensions is most successful with the adenocarcinoma cell lines, Caco-2 and T-84, and the human liver hepatoma cell line, PLC/PRF/5 (Mendez and Arias 2007). Virus detection is carried out mainly by EM of stool specimens, diagnostic RT-PCR using broadly reactive primers, or by an integrated cell culture-RT-PCR (ICC-RT-PCR) procedure. Astroviruses are resistant to extreme environmental conditions. Their heat tolerance allows them to survive 50 °C for 1 h. At 60 °C, the virus titer falls by 3 $\log_{10}$ and 6 $\log_{10}$ after 5 and 15 min, respectively. Astroviruses are stable for years at ≤ −70 °C but repeated freeze/thawing cycles can lead to structural damage. The virus is stable at pH 3.0 and is resistant to chemicals, including chloroform, lipid solvents, and alcohols and to non-ionic, anionic and Zwitter ionic detergents. Astrovirus particles exhibit a range of buoyant densities in cesium chloride (CsCl), from 1.35 to 1.39 g/mL for complete virus particles, and from 1.28 to 1.33 g/mL for empty (no genome) capsids or VLPs. Divalent cations (Ca^{2+} and Mg^{2+}) in CsCl facilitate virus and VLP stabilization (Mendez and Arias 2007).

7.4. Infection and Disease

Clinically, astroviruses cause symptoms similar to those of caliciviruses following an incubation period of 3–4 days although shorter incubation periods (24–36 h) have also been reported. Symptoms include diarrhea, fever, nausea, and general malaise with occasional vomiting. Normally, diarrhea persists for only 2–3 days but can be prolonged for up to 14 days with virus excretion in feces. High concentrations of astrovirus (up to 10^{10} or 10^{11} viruses per gram) can be shed in feces (Mendez and Arias 2007). Severe illnesses and death can occur but are rarely reported. Outbreaks commonly occur in institutional settings, especially pediatric wards. In temperate climates, a seasonal peak in winter and spring occurs, but infections may occur throughout the year, especially during the rainy season in tropical climates.

7.5. Foodborne Disease

Epidemiological evidence of transmission by foods is limited but infections via contaminated shellfish and water have been reported (Appleton 2001; Oishi et al. 1994). In 1991, a large outbreak of acute gastroenteritis occurred in Japan involving thousands of children and adults from 14 schools (Oishi et al. 1994). The outbreak was traced to food prepared by a common supplier for school lunches; astrovirus type 6 was identified by immune electron microscopy and confirmed by molecular and culture methods. There are several reports of astrovirus genomes identified in shellfish and evidence indicates that astroviruses may contribute to outbreaks of gastroenteritis through

consumption of contaminated oysters (Kitahashi et al. 1999; Le Guyader et al. 2008; Ming et al. 2013). In a French shellfish-related gastroenteritis outbreak, astroviruses were among a range of enteric viruses identified in patient feces and related shellfish samples (Le Guyader et al. 2008). Given the wide range of animal astroviruses discovered in recent years and their capacity for genetic mutation and recombination, interspecies replication and zoonotic transmission is possible although, to date, no zoonotic astrovirus strains have been reported in humans (Bosch et al. 2014).

8. OTHER VIRUSES WITH POTENTIAL FOR FOODBORNE TRANSMISSION

Other viruses transmitted by the fecal-oral route and found in feces of humans and animals include the adenoviruses, enteroviruses, aichiviruses, parvoviruses, coronaviruses, toroviruses, picobirnaviruses, polyomaviruses and the tick borne encephalitis virus (Table 2.1). Some of these viruses have caused a small number of foodborne outbreaks in the past. While they are currently considered minor foodborne pathogens, there is a possibility that more virulent or transmissible strains of these viruses might emerge making them pathogens of significance. For some of these viruses, the ability to cause illness in humans and or animals is still unproven. Readers are referred to a published review providing more description on these viruses and their potential to cause foodborne disease (Duizer and Koopmans 2008). A brief description of these viruses is given below.

8.1. Adenoviruses

Adenoviruses belong to the *Adenoviridae* family and are classified into five genera, *Mastedenovirus* (infect mammals), *Aviadenovirus* (infect birds), *Atadenovirus* (infect sheep, cattle, ducks and possum), *Ichtadenovirus* (infects sturgeon) and *Siadenovirus* (infect reptiles and birds). Adenoviruses are 80–110 nm non-enveloped, linear double-stranded DNA viruses with icosahedral symmetry and a genome of 28–45 kb (Table 2.1, Fig. 2.1c). Most human adenovirus (HAdV) infections in normally healthy individuals are mild or subclinical but can be associated with respiratory, ocular and gastrointestinal disease. HAdV-F types 40 and 41 are generally associated with fecal-oral spread and cause gastroenteritis, although all types are shed in feces, often in large numbers (up to 10^{11} particles/g feces) for several months (Wold and Horwitz 2007). Over 60 human adenovirus types have been recognized that appear to be specific to humans as none have been detected in the stools of domestic and wild mammals (Jiang 2006). This feature makes them a good candidate for microbial source tracking to identify sources of fecal pollution (Wong et al. 2012).

The adenoviruses are capable of prolonged survival in the environment and are considered to be more stable than other enteric viruses and faecal indicator bacteria in many environmental situations. The A549 and 293 cell lines have been used successfully for the isolation of adenoviruses from food and environmental samples, although members of the F subgroup are more fastidious than other human adenoviruses. Adenoviruses can be transmitted person-to-person by direct contact or via fecal-oral, respiratory or environmental routes. Waterborne transmission of adenovirus has been associated with conjunctivitis in children. Enteric adenovirus infections are common all year round, whereas outbreaks of adenovirus-associated respiratory disease normally occur from late winter to early summer. Adenoviruses have been detected in a variety of environmental samples, including wastewater, sludge, shellfish, and marine, surface and drinking waters. No foodborne or waterborne outbreaks associated with the enteric adenoviruses have been reported but, as these viruses are common in the environment, it is possible that disease has occurred but the source of infection was not recognized. There are no reports of foodborne transmission or disease resulting from consumption of adenovirus-contaminated shellfish.

8.2. Enteroviruses

The enteroviruses are 28–30 nm non-enveloped, positive-sense single-stranded RNA viruses with icosohedral symmetry and a genome of 7.2–8.4 kb (Table 2.1). They are classified in the large *Picornaviridae* family and 12 species are currently designated within the *Enterovirus* genus, including Enterovirus A, B, C, D, E, F, G, H, and J (the letter I was skipped) and Rhinovirus A–C (ICTV 2012; Knowles et al. 2011b). Enteroviruses include polioviruses, coxsackie A and B viruses, echoviruses, and rhinoviruses, many of which are culturable. Enteroviruses cause a range of diseases, including viral meningitis and poliomyelitis. They multiply mainly in the gastrointestinal tract but can also multiply in other tissues such as nerve and muscle, as does the poliovirus. The incubation period is usually between 3 and 7 days with virus transmission occurring from 3 to 10 days after symptoms develop. Enteroviral infection is most common in summer and early autumn and many infections are asymptomatic. Only a few people (approximately 0.001 %) develop aseptic or viral meningitis and no long-term complications normally follow the mild illnesses or aseptic meningitis. On rare occasions, a person may develop myocarditis or encephalitis.

Many enteroviruses are transmitted by the fecal-oral route and are excreted in feces but do not generally cause gastroenteritis. Because they are easily cultured *in vitro* and are stable in the environment, live attenuated vaccine strains of poliovirus have been used as indicator viruses for the presence of other virulent enteric viruses in food and water. They have also been used extensively in environmental and food virology research for method development and to gather information on virus recovery, persistence and behavior in these settings. The first recorded outbreak associated with foodborne viruses was an outbreak of poliomyelitis linked to consumption of raw milk in

1914 (Jubb 1915). A further ten outbreaks associated with raw milk consumption were reported in the USA and United Kingdom over the following 35 years (Sattar and Tetro 2001). The widespread introduction of pasteurized milk in the 1950s decreased transmission by this route. There have been few recorded foodborne outbreaks associated with enterovirus infection despite the regular occurrence of enteroviruses in the environment. Enteroviruses, including echoviruses and coxsackie A and B viruses, have been isolated from sewage, raw and digested sludge, marine and fresh waters, and shellfish. In two reported foodborne outbreaks associated with echoviruses in the United States, the source of the virus was not identified (Cliver 1997). Although enteroviruses are often present in shellfish, other enteric viruses are also usually present and no shellfish—associated outbreaks have been reported specifically for enteroviruses.

8.3. Aichivirus

Aichivirus A (formerly Aichi virus) is a single stranded RNA virus classified in the Genus *Kobuvirus*, in the Family *Picornaviridae*. It was first discovered in 1989 in Japan by Yamashita et al. (1991) as the cause of oyster-associated gastroenteritis (Yamashita et al. 1991). It was later identified in gastroenteritis cases from several Asian countries and classified as a novel picornavirus (Yamashita et al. 1993; Yamashita et al. 1998). Aichiviruses have been detected in stools, human sewage and in shellfish worldwide, but seem to be more often detected in Asian countries. Aichivirus was identified in 33 % of 57 commercial packages of Japanese clams (Hansman et al. 2008). The first identifications of the virus outside Asia occurred in clinical specimens in Germany and Brazil (Oh et al. 2006). Aichiviruses were also among several enteric viruses identified in clinical specimens and oysters associated with a gastroenteritis outbreak in France (Le Guyader et al. 2008). Since it is often detected along with other viruses associated with gastrointestinal illness, its importance as a food- and water-borne pathogen is still unclear. The virus is culturable and produces cytopathic effects in B-SC 1 cells.

8.4. Parvovirus

Parvoviruses have been proposed as causal agents of human gastroenteritis but their role in viral gastroenteritis of some animal species has been well documented. They are single stranded DNA viruses classified in the family *Parvoviridae*, subfamily Parvovirinae, genus *Erythrovirus*, which includes type species human parvovirus B19. They are among the smallest known viruses at 18–26 nm in diameter. Parvoviruses have a smooth surface with no discernable features and were included under 'small round viruses' before definitive classification of these viruses was completed. Three possible serotypes known as the Parramatta agent, the cockle agent and the Wollan/Ditchling group have been identified by immune electron microscopy. There

is limited evidence of parvovirus association with foodborne disease but it has been linked with consumption of contaminated shellfish (Appleton 2001; Appleton and Pereira 1977). The 'cockle agent' parvovirus was implicated in a large UK outbreak related to consumption of contaminated cockles (Appleton 2001). Over 800 confirmed cases of gastroenteritis occurred and parvovirus was identified in all stools examined from this large gastroenteritis outbreak.

Human bocaviruses (1, 2 a–c, 3 & 4) are also classified in the *Parvoviridae* family (subfamily *Parvovirinae*, *Bocaparvovirus* genus). Although they have been detected in feces and waters and may be transmitted by the fecal-oral route, bocavirus type 1 is mainly associated with pediatric respiratory diseases (Arthur et al. 2009) and the role of bocavirus type 2 in gastroenteritis is currently unclear (Chhabra et al. 2013; Chow et al. 2010).

8.5. Coronavirus

Coronaviruses are large (80–220 nm), pleomorphic, enveloped, positive sense single-stranded RNA viruses belonging to the *Coronaviridae* family (human strains reside in *Alphacoronavirus* and *Betacoronavirus* genera). They generally cause respiratory infections but can also cause gastroenteritis in animals and are excreted in feces. Their role in human gastroenteritis is unclear, although 'coronavirus-like particles' can be identified in human feces (Glass 1995). SARS (severe acute respiratory syndrome) and the MERS (Middle East respiratory syndrome) coronaviruses (CoVs) have been associated with severe atypical pneumonia and numerous deaths since their emergence in 2002 and 2012, respectively. Bats appear to be the primary reservoir for both of these viruses. However, intermediate hosts appear to be responsible for zoonotic transmission to humans. SARS-CoV infections in humans have been linked to palm civets and raccoon dogs sold at Chinese exotic markets (Graham and Baric 2010). Dromedary camels may be associated with human infections by MERS-CoV in the Middle East, as evidenced by neutralizing antibodies and positive nasal swabs detected in these animals (Milne-Price et al. 2014); however, a direct evidence of zoonotic transmission has not been reported to date.

8.6. Torovirus

Toroviruses are 100–150 nm, enveloped, positive sense, single-stranded RNA viruses belonging to the Genus *Torovirus* in the *Coronaviridae* family. They were first discovered in 1979 and were named as Breda viruses (Glass 1995; Woode et al. 1982). When observed by EM they have a distinctive pleomorphic appearance with club-shaped projections extending from the capsid. Toroviruses are known to cause gastroenteritis in animals, especially dairy cattle, in which they cause a marked decrease in milk production. Although

they have been isolated from feces of children and adults with diarrhea (Koopmans et al. 1991), their exact role in human gastroenteritis and foodborne disease is still unknown.

8.7. Picobirnavirus

The picobirnaviruses are small non-enveloped 33–41 nm positive sense double-stranded RNA viruses with a segmented genome and are classified in the family *Picobirnaviridae*, genus Picobirnavirus (Knowles et al. 2011a). These viruses have been detected in feces of many animals and birds and are known to cause gastroenteritis in a range of domestic animals. They have also been detected in humans with and without diarrhea and are now regarded as opportunistic pathogens which can cause gastroenteritis in immunocompromised patients. They have been detected in sewage and river waters (Hamza et al. 2011) and in humans from several countries, including Australia, Brazil, England and the USA. Their role as true human pathogens is unproven and although there is no documented evidence of foodborne transmission, it is likely that they can be transmitted by the fecal-oral route and possibly via food.

8.8. Tick-Borne Encephalitis Virus

Tick-borne encephalitis (TBE) virus along with other closely related arboviruses that cause yellow fever, Japanese encephalitis, dengue fever and West Nile disease are enveloped, positive sense single- stranded RNA viruses classified in the *Flaviviridae* family under the Flavivirus genus. Tick-borne encephalitis is a zoonotic arbovirus infection endemic to Eastern and Central Europe and Russia. However, the distribution of these viruses can extend to Northern Europe, China, Japan and Korea. Three subtypes of TBE virus cause tick-borne encephalitis: the Eastern European subtype, the Western European subtype and the Siberian subtype. Most cases occur in spring and summer following bites of different species of *Ixodes* tick. Foodborne transmission is less common but can occur following consumption of unpasteurized dairy products such as milk, yogurt, butter and cheese from infected cattle and goats (Döbbecker et al. 2010). The disease is serious and can result in long-term neurological sequelae or death. Increased tourism to the endemic areas has extended the risk of travellers acquiring TBE. Vaccines are now available in several countries.

8.9. Other Foodborne Routes of Virus Transmission

Transmission of viral disease can occur through human breastfeeding especially some of the bloodborne viruses. There are reports of human immunodeficiency virus (HIV), human lymphotrophic virus -1 (HTLV-1), hepatitis B virus and cytomegalovirus (CMV) being transmitted to infants in milk from

infected mothers during breast feeding (Pol et al. 2011; Sattar and Tetro 2001). This can present serious problems in less developed countries with a high incidence of HIV and few alternative options for feeding available to infected mothers.

9. SUMMARY AND CONCLUSIONS

Foodborne disease is increasing worldwide and has become a major public health problem. A majority of foodborne viral disease is caused by noroviruses and HAV. Foodborne transmission of other enteric viruses is less common. The HEV is the only virus that appears to be a likely candidate for direct zoonotic transmission from animals to man, although to date there have been few reports of its transmission by this route.

The overall contribution of viruses to global foodborne disease burden is unknown because accurate data on the prevalence of foodborne viral disease are not available for all countries. National epidemiological surveillance systems vary from country to country and a large proportion of viral infections are not notifiable and therefore not reported. However, from epidemiological data that has been collected in Europe, the USA and other countries, it is apparent that viruses play a significant role and that the economic burden of foodborne viral gastrointestinal disease can be substantial in terms of staff illness, time away from employment and disruption to services.

Over the last 20 years, the introduction of molecular methods for detection and identification of enteric viruses, many of which are not culturable or are difficult to grow in culture, has greatly increased our understanding of their role in foodborne disease. The development of techniques, such as PCR and real-time quantitative PCR, is rapidly increasing the knowledge base by facilitating studies on the behavior and persistence of these viruses in food matrices. However, it is important to recognize the limitations of these techniques. PCR-based methods currently detect both infectious and non-infectious viruses and are not able to measure viral infectivity, which is the key factor when assessing human health risks from foodborne pathogens.

Recently, several novel molecular—based methods have been developed to determine viral infectivity in non-culturable viruses, but none have yet been validated for regulatory or epidemiological use (Knight et al. 2013). The overall strategies used in these methods are either assessment of virus capsid integrity by receptor or antibody binding, or use of nucleic acid-intercalating chemicals or RNases to disrupt RNA such that only intact viral RNA is amplified by RT-PCR. Sometimes longer portions of the genome are targeted to better assess RNA damage. If the virus is no longer detectable in molecular assays after employing these methods, it is considered to be non-infectious.

It is important however that judicious interpretation of data generated solely from molecular-based assays is used when studying these viruses. Use of integrated cell culture combined with PCR methods (ICC-PCR) can

overcome some of the problems associated with viruses that are difficult to grow. Unfortunately, the infectivity status of the main foodborne viral pathogen, norovirus, still cannot be determined routinely by *in vitro* methods. This has limited our knowledge of the natural history and biological properties of this pathogen and has also slowed progress in the development of effective control and intervention strategies.

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CHAPTER 3

The Molecular Virology of Enteric Viruses

Javier Buesa and Jesús Rodriguez-Díaz

1. CALICIVIRUSES: NOROVIRUSES AND SAPOVIRUSES

The *Caliciviridae* family is currently divided into five genera: *Norovirus*, *Sapovirus*, *Lagovirus*, *Vesivirus* and *Nebovirus* (King et al. 2012). Two other potential genera, *Valovirus* and *Recovirus*, have also been described (L'Homme et al. 2009b). The prototype norovirus, the Norwalk virus, was first described in 1972 as the etiological agent of an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio (Kapikian 2000). Subsequently, Noroviruses (NoV) were found to be the cause of a majority of outbreaks of acute nonbacterial gastroenteritis. The NoVs are now recognized as a very common cause of sporadic cases of diarrhoea in the community (Glass et al. 2000b; Lopman et al. 2002; Hutson et al. 2004; Estes et al. 2006) and were found to be responsible for as many as 95 % of the reported viral gastroenteritis outbreaks over a 4.5-year period in the U.S. (Fankhauser et al. 2002). Similarly high percentages have also been reported from other industrialized countries (Maguire et al. 1999; Glass et al. 2000b; Koopmans et al. 2000; Lopman et al. 2003).

Common features of members of *Caliciviridae* include the presence of a single major structural protein from which the capsid is constructed and the appearance of 32 cup-shaped depressions on the surface of the virion arranged in an icosahedral symmetry. The name of the family was derived from the Latin word *calix,* which means cup or goblet, and refers to the surface hollows on the virion (Madeley 1979). Another major feature is the absence of a methylated cap structure or a ribosomal entry site (IRES) at the 5′ end of the viral RNA. Instead, a small protein (VPg) of ~10–12 kDa was shown to be covalently linked to the viral RNA and was described as essential for the infectivity of the RNA (Black et al. 1978). This has also been described for other caliciviruses (Burroughs and Brown 1978). The VPg protein interacts with components of the translation machinery (eIF3, eIFGI, eIF4E, and S6 ribosomal protein) and may play a role in initiating translation of NoV RNA (Daughenbaugh et al. 2003). The linkage of VPg to viral RNA is thought to occur during viral genome replication whereby VPg is attached as a protein primer to the 5′ terminus of the genomic RNAs (Rohayem et al. 2006).

The noroviruses and sapoviruses form distinct phylogenetic clades within the *Caliciviridae* family (Berke et al. 1997). In addition, certain features of their viral genome organization distinguish them from each other and from

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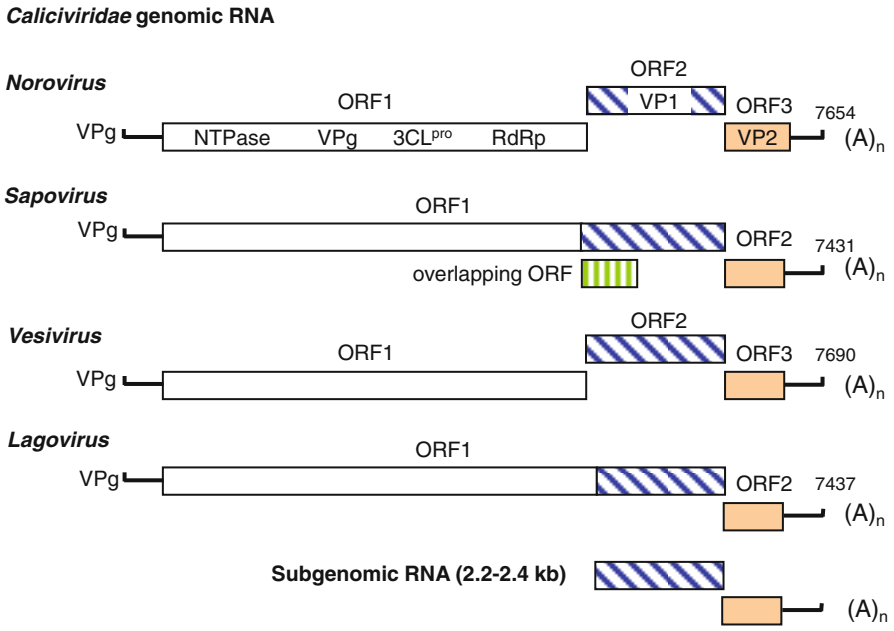

Figure 3.1. Genome organization of the four different genera of Caliciviridae. The genome of Norovirus and Vesivirus has three open reading frames (ORFs) that encode the nonstructural proteins, the major capsid protein (VP1), and a minor structural basic protein (VP2). The genera Sapovirus and Lagovirus encode the capsid protein contiguous with the large nonstructural polyprotein (ORF1). An additional small overlapping ORF in a +1 frameshift has been described in certain strains of sapoviruses. A subgenomic RNA that covers the entire 3′ end of the genome, from the capsid gene to the 3′ end, has been detected in calicivirus infected cells.

other genera of *Caliciviridae* (Fig. 3.1). Noroviruses and sapoviruses also differ in their epidemiology and host range. For example, NoVs can infect individuals of all ages and are commonly involved in outbreaks of acute gastroenteritis frequently associated with contaminated food or water. Sapoviruses, on the other hand, mainly infect infants and young children (Green et al. 2001) although gastroenteritis outbreaks in adults have also been described (Noel et al. 1997).

The NoVs have a polyadenylated positive-sense single-stranded RNA genome with three major open reading frames (ORFs) (Jiang et al. 1993b; Lambden et al. 1993). The virion has a buoyant density of 1.33–1.41 g/cm^3 in cesium chloride (CsCl) (Caul and Appleton 1982; Madore et al. 1986) and usually lacks the distinctive calicivirus cup-like morphology when viewed under electron microscope (EM). Noroviruses were formerly referred to as small round structured viruses (SRSVs). The sapoviruses, associated with sporadic cases of acute gastroenteritis, have a polyadenylated positive-sense

single-stranded RNA genome (7.3–7.5 kb in length) with two main ORFs (Liu et al. 1995). The virions have a buoyant density of 1.37–1.38 g/cm^3 in CsCl (Terashima et al. 1983), and often possess distinctive calicivirus cup-like morphology when viewed under EM (Madeley 1979). Sapoviruses ("classic" caliciviruses) are important enteric pathogens that can cause diarrhea in humans, pigs and mink (Guo et al. 1999b; Guo et al. 2001b; Martella et al. 2008).

Animal enteric caliciviruses are an important cause of gastroenteritis in domestic animals, namely calves and pigs (Liu et al. 1999; Saif et al. 1980; Bridger et al. 1984; van Der Poel et al. 2000; Guo et al. 2001a; Scipioni et al. 2008). Murine noroviruses (MNVs) have been isolated from both immunodeficient and immunocompetent laboratory mice (Karst et al. 2003; Hsu et al. 2006), but their pathogenicity is rather different from that caused by human noroviruses.

Despite numerous attempts, human noroviruses had not been propagated successfully in cultured cells until recently (Jones et al. 2014), thus hampering many aspects of research (Duizer et al. 2004; Herbst-Kralovetz et al. 2013; Papafragkou et al. 2013). However, considerable progress has been made by the analysis of cDNA clones generated from the genomic RNA of virions in stool material. In fact, the molecular era of norovirus research started with the successful cloning of the genomes of Norwalk and Southampton viruses from stool samples (Jiang et al. 1990; Lambden et al. 1993). Unlike human caliciviruses, some animal caliciviruses have been successfully propagated in cell cultures including primate calicivirus (Smith et al. 1983), feline calicivirus (FCV) (Love and Sabine 1975), and San Miguel sea lion virus (SMSV) (Smith et al. 1973). These viruses have provided a direct approach for the study of virus infections, genome transcription, viral protein translation, and virus replication (Green et al. 2002). In addition, information gained by the study of caliciviruses that grow efficiently in cell culture, such as FCV and vesicular exanthema of swine virus (VESV), or that have an animal model and a limited cell culture system such as rabbit hemorrhagic disease virus (RHDV), has been important for the identification of features that are likely to be shared among members of the *Caliciviridae* family (Marin et al. 2000; Morales et al. 2004).

Thus, the murine norovirus (MNV) revealed an unexpected tropism for hematopoietic cell lineages, particularly dendritic cells and macrophages (Wobus et al. 2004). The MNV can be grown in vitro; the RAW264.7 cell line has been used to design a typical virus plaque assay and to grow plaque-purified viral isolates (Mumphrey et al. 2007). The MNV has offered the first chance to study the entire norovirus replication cycle in the laboratory. Despite differences in diseases caused by human and murine NoVs, the MNV possesses a high genetic similarity and constitute an excellent model system to study the mechanisms of NoV translation and replication, as well as pathogenesis and immunity (Vashist et al. 2009). More recently, Jones et al. showed that human noroviruses could be propagated in the human B lymphocyte derived cell line BJAB (Jones et al. 2014). These experiments confirm the tropism of noroviruses for hematopoietic cell lineages. Another relevant aspect of norovirus

infections is the role of gut microbiota as a cofactor needed for efficient virus-cell attachment and infection, as it has been demonstrated "in vitro" for human noroviruses (Miura et al. 2013; Rubio-del-Campo et al. 2014; Jones et al. 2014) and "in vivo" for murine norovirus (Jones et al. 2014; Baldridge et al. 2015).

1.1. Structure and Composition

The Norwalk virus capsid is composed of a single major structural protein known as VP1, and a few copies of a second small basic structural protein named as VP2 (Prasad et al. 1999; Glass et al. 2000a; Green et al. 2001). The characteristic cup-shaped structures on the surface of the virions are more prominent in some strains, particularly in sapoviruses, leading to the characteristic six-pointed "star of David" appearance when viewed along the major two-, three- and fivefold axes of symmetry. Cloning and expression of norovirus proteins VP1 and VP2 in insect cells using the baculovirus expression system resulted in the self-assembly of the viral capsid and the production of recombinant virus-like particles (rVLPs) that were antigenically and structurally similar to native virions (Jiang et al. 1992; Green et al. 1997; Hale et al. 1999; Kobayashi et al. 2000).

The three-dimensional structure of Norwalk rVLPs was first determined by cryo-electron microscopy and computer image processing to a resolution of 22 Å. This analysis showed that the virus particles (38 nm in diameter by this technique) have a distinct architecture and exhibit T = 3 icosahedral symmetry (Fig. 3.2). The capsid contains 180 copies of the capsid protein assembled into 90 dimers with an arch-like structure. The arches are arranged in such a way that there are large hollows at the icosahedral five- and threefold positions; these hollows are seen as cuplike structures on the surface of caliciviruses (Prasad et al. 1994; Prasad et al. 1996a; Prasad et al. 1999). To form a T = 3 icosahedral structure, the capsid protein has to adapt to three quasi-equivalent positions; the subunits located at these positions are conventionally referred to as A, B and C. The high resolution (3.4 Å) structure of the Norwalk virus capsid has been determined by X-ray crystallography (Prasad et al. 1999).

Each subunit or monomeric capsid protein folds into an N-terminal region facing the inside of the capsid, a shell-domain (S) that forms the continuous surface of the VLP, and a protruding (P) domain that forms the protrusions (Fig. 3.2). A flexible hinge of eight amino acids links the S and P domains. The P domain is located at the exterior of the capsid and is likely to contain determinants of genotype specificity. The NH_2-terminal (N) arm, located within the S domain, consists of residues 10–49 and faces the interior of the capsid. The part of the S domain that forms a ß-barrel consists of amino acids 50–225. The entire S domain (amino acids 1–225) corresponds to the N-terminal region of the capsid protein that is relatively conserved among "Norwalk-like viruses" in sequence comparisons. Amino acid residues 226–530 form the P domain, which corresponds to the C-terminal half of the capsid protein and forms the arch-like structures extending from the shell. The S domain is

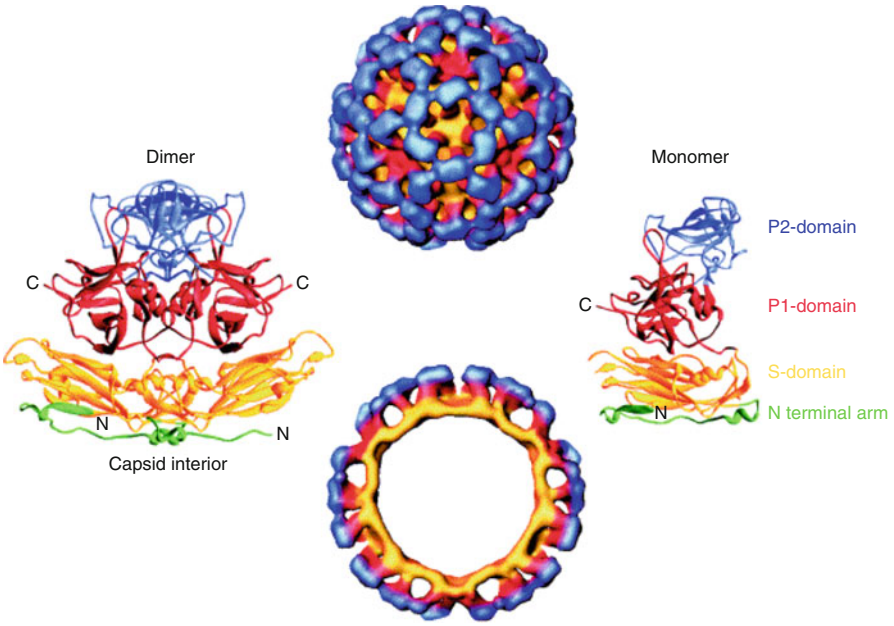

Figure 3.2. The structure of Norwalk virus-like particles (NV VLPs) has been solved by cryo-electron microscopic reconstruction to 22 Å (top, surface representation; bottom, cross section) and by x-ray crystallography to 3.4 Å. The NV VLPs have 90 dimers of capsid protein (*left*, ribbon diagram) assembled in T = 3 icosahedral symmetry. Each monomeric capsid protein (*right*, ribbon diagram) is divided into an N-terminal arm region (*green*) facing the interior of the VLP, a shell domain (S domain, *yellow*) that forms the continuous surface of the VLP, and a protruding domain (P domain) that emanates from the S domain surface. The P domain is further divided into subdomains, P1 and P2 (*red* and *blue*, respectively) with the P2 subdomain at the most distal surface of the VLPs (Reproduced with permission from Prasad et al. 1999, and Bertolotti-Ciarlet et al. 2002).

required for the assembly of the capsid and also participates in multiple intermolecular interactions of dimers, trimers and pentamers.

The P domain is mainly involved in dimeric interactions only (Prasad et al. 1999) and has two subdomains: P1 formed by amino acids 226–278 and 406–530, and P2 encompassing amino acids 279–405. The P2 subdomain is the most variable region of the capsid protein among NoVs (Hardy et al. 1996) and plays an important role in immune recognition and receptor interaction. It has been shown that isolated P domains form dimers and bind to histo-blood group antigens (HBGA) without requiring the formation of VLPs (Tan et al. 2004). In addition, a binding pocket in the P domain is responsible for viral HBGA-binding and the formation of this pocket involves only intramolecular interactions (Tan et al. 2003). Although the S domain has a canonical 8-stranded β-barrel structure, the P2 subdomain has a fold similar to that observed in

domain 2 of the elongation factor Tu (EF-Tu), a structure never seen before in a viral capsid protein (Prasad et al. 1999). Moreover, the fold of the P1 subdomain is unlike any other polypeptide observed so far (Bertolotti-Ciarlet et al. 2003).

A comparison of the capsid proteins from various caliciviruses reveals significant variations in their sequences and sizes. In general, the capsid proteins of human caliciviruses are smaller than those of animal caliciviruses (Chen et al. 2004). The Norwalk virus recombinant capsid protein can also self-assemble into smaller VLPs (23 nm) with suspected T = 1 symmetry that are thought to be composed of 60 copies of the capsid protein (White et al. 1997).

It has been postulated that the N-terminal region of the capsid protein or the genomic RNA acts as a switching region that controls variations in the conformation of the coat protein of the T = 3 viruses (Rossmann and Johnson 1989). NoV particles are different from other T = 3 viruses; their recombinant capsid protein readily forms rVLPs without RNA (Prasad et al. 1999). It has been suggested that the determinants for the T = 3 capsid assembly for Norwalk virus may lie outside of the N-terminus and that the interaction between the subunits B and C is not mandatory for the formation of the capsid (Bertolotti-Ciarlet et al. 2002).

1.2. Genomes and Proteins

As mentioned above, all caliciviruses have a linear, single-stranded, positive-sense RNA genome of 7.5–7.7 kb (Green et al. 2001) (Fig. 3.1). The RNA genome of Norwalk virus, the prototype strain for the genus *Norovirus*, is 7654 nucleotides in length and is polyadenylated at the 3′ end (Jiang et al. 1993b). The lack of a cap structure typical of eukaryotic mRNA and the absence of an internal ribosomal entry site suggest that the VPg protein may function in translation initiation through unique protein-protein interactions with the cellular translation machinery (Daughenbaugh et al. 2003). The genomes of caliciviruses are organized into either two or three major ORFs with the exception of MNV, which has a fourth alternative ORF (McFadden et al. 2011). The nonstructural proteins encoded in the calicivirus ORF1 were first predicted based on sequence similarities with picornavirus nonstructural proteins (Neill 1990). Amino acid sequence motifs in common with the poliovirus 2C NTPase, 3C protease, and 3D RNA-dependent RNA polymerase (RdRp) were readily identified and provided templates for further characterization of the calicivirus nonstructural proteins. Proteolytic mapping and enzymatic studies of in vitro-translated polyprotein or recombinant protein expression has confirmed the presence of an NTPase (p41), a 3C-like protease ($3CL^{pro}$), an RdRp, and the location in the polyprotein of the genome-linked protein VPg (Liu et al. 1996; Dunham et al. 1998; Pfister and Wimmer 2001). The proposed six nonstructural proteins encoded in the norovirus ORF1 defined so far proceed from N to C terminus, p48-NTPase-p22-VPg-$3CL^{pro}$-RdRp (Ettayebi and Hardy 2003). It has been reported that the 3C-like proteinase ($3CL^{pro}$) inhibits host cell translation by cleavage of poly(A)-binding protein (PABP), which is a key protein involved in the translation of

polyadenylated mRNAs (Kuyumcu-Martinez et al. 2004). Functional analysis of the protein produced from MNV ORF4 demonstrated that it antagonizes the innate immune response to infection by delaying the upregulation of a number of cellular genes activated by the innate pathway, including IFN-beta (McFadden et al. 2011).

In the genera *Norovirus* and *Vesivirus*, the capsid structural protein VP1 is encoded in a separate ORF (ORF2), whereas the capsid proteins encoded in *Sapovirus* and *Lagovirus* genera are contiguous with the large nonstructural polyprotein (ORF1) (see Fig. 3.1). Viruses in the genera *Sapovirus* and *Lagovirus* have two major ORFs (ORF1 and ORF2). In the *Sapovirus* genera, the ORF1/2 junction consists of a one- or four-nucleotide overlap between the stop codon of ORF1 and the first AUG codon of ORF2. A third ORF has been described in certain strains that overlaps with the capsid protein gene in a +1 frameshift and is not found in the *Norovirus* genome (Liu et al. 1995; Clarke and Lambden 2000). The presence of a conserved translation initiation motif GCAAUGG at the 5′ end of this overlapping ORF suggests that a biologically active protein may be encoded in this ORF (Schuffenecker et al. 2001).

Viruses in the genera *Norovirus* and *Vesivirus* have three major ORFs (ORF1, ORF2, and ORF3). In the noroviruses the first and third ORFs are in the same reading frame. In Norwalk virus, ORF3 encodes a 212-amino-acid minor structural protein of the virion (Glass et al. 2000a). All calicivirus genomes begin with a 5′-end terminal GU, which is repeated internally in the genome and most likely correspond to the beginning of a subgenomic-sized RNA transcript (2.2–2.4 kb) that is co-terminal with the 3′ end of the genome. This has been observed in FCV- and RHDV-infected cells as well as in packaged virions (Herbert et al. 1996a). A comparison of the 5′-end sequences of representative viruses within each of the four genera and the corresponding repeated internal sequence suggests this feature is characteristic of the caliciviruses. The synthesis of a subgenomic RNA in calicivirus-infected cells is a major difference between the replication strategy of caliciviruses and picornaviruses, although several of the replicative enzymes share distant homology (Green et al. 2001). Calicivirus structural proteins are expressed from a subgenomic mRNA with two overlapping cistrons. The first ORF of this RNA codes for the major capsid protein VP1, while the second codes for the minor capsid protein VP2. Translation of VP2 is mediated by a termination/reinitiation mechanism, which depends on an upstream sequence element of approximately 70 nucleotides denoted "termination upstream ribosomal binding site" (TURBS). Two short sequence motifs within the TURBS were found to be essential for reinitiation (Luttermann and Meyers 2009).

By functional analysis of the 5′ genomic sequence of Jena virus, a bovine norovirus, the N-terminal encoding genomic sequence was tested for IRES-like function in a bi-cistronic system but displayed no evidence of IRES-like activity (Salim et al. 2008). Alignment of the N-terminal protein sequences of various noroviruses showed little similarity between genogroups within the first 180 residues; however, towards the C-terminal end of the protein,

similarity between the amino acid residues increased. Recent studies investigating the functions of the Norwalk virus N-terminal protein have successfully demonstrated its association with the Golgi apparatus in transfected cells (Fernandez-Vega et al. 2004). Other studies have suggested that the Norwalk virus N-terminal protein disrupts intracellular protein trafficking, including proteins destined for the host cell membrane (Ettayebi and Hardy 2003). A 3C protease-mediated cleavage event within the N-terminal protein (37 kDa) was described for Camberwell virus, a genogroup 2 norovirus, yielding proteins of 22 and 15 kDa (Seah et al. 2003). Based on these observations and location within the genome it was hypothesised that the N-terminal protein of noroviruses corresponds to the 2AB region in picornaviruses. After expression of the structural proteins from subgenomic RNA molecules, the capsid is assembled and viral RNA is encapsidated prior to progeny release. Some of these features have been confirmed using recombinant systems to express the native Norwalk virus genomes in mammalian cells using vaccinia virus expression systems (Asanaka et al. 2005; Katayama et al. 2006).

1.3. Molecular Diversity of Noroviruses

Early studies demonstrating variability in NoVs soon led to the notion that it was important to distinguish between strains to better understand their epidemiology. Since no antigenic analysis of norovirus strains was available due to the lack of immune reagents, genome characterization by sequence analysis has been used to provide an interim system of genotyping (Koopmans et al. 2003). As the genotypes ideally would correlate with serotypes, the amino acid sequence of the major structural protein was used as the basis for phylogenetic analyses (Ando et al. 2000; Koopmans et al. 2001). Noroviruses encompass six distinct genogroups (GI-GVI) with GI, GII and GIV infecting humans, which are further divided into >30 genotypes (Zheng et al. 2006; Vinjé 2015) (Table 3.1). Genogroups were defined on the basis of amino acid diversity of the 3 ORFs, the RdRp and VP1 encoding regions, or the VP1 encoding region alone. Current classification into six genogroups is based on the VP1 protein divergence (Green et al. 2000; Vinjé et al. 2004; Zheng et al. 2006; Vinjé 2015). Genotypes are defined on the basis of either the polymerase or the capsid sequence, assuming that the genotype defined by one region of the genome would correspond to the whole genome because of the non-segmented nature of the norovirus genome (Vinjé and Koopmans 1996; Kageyama et al. 2004; Vinjé et al. 2004). New genotypes were assigned when VP1 amino acid sequence differed by more than 20 % with other known genotypes (Vinjé et al. 2000). However, with the accumulation of more norovirus sequence data, this cutoff threshold has been changed to 14.1 % and a minimum of 15 % pairwise difference between the next-nearest genotype for the proposal of a new norovirus genotype (Zheng et al. 2006; Kroneman et al. 2013).

The recognition that recombination often occurs at the ORF1/ORF2 junction, has necessitated the determination of both polymerase and capsid gene

Table 3.1 Current genogroups and genotypes of noroviruses (adapted from Green et al. (2001), Koopmans et al. (2003) and Vinjé (2015))

Genogroup	*Genotype*	*Prototype strain*[a]	*GenBank accession number (capsid gene)*	*Other strains*
I	1	Norwalk/1968/UK	M87661	KY/89/JP
	2	Southampton/1991/UK	L07418	White Rose, Crawley
	3	Desert Shield/395/1990/SA	U04469	Birmingham 291
	4	Chiba 407/1987/JP	D38547	Thistle Hall; Valetta, Malta
	5	Musgrove/1989/UK	AJ277614	Butlins
	6	Hesse 3/1997/DE	AF093797	Sindlesham, Mikkeli, Lord Harris
	7	Winchester/1994/UK	AJ277609	Lwymontley
	8	Boxer/2001/US	AF538679	
	9	Vancouver730/2004/CA	HQ637267	
II	1	Hawaii/1971/US	U07611	Wortley, Girlington
	2	Melksham/1994/UK	X81879	Snow Mountain
	3	Toronto 24/1991/CA	U02030	Mexico, Auckland, Rotterdam
	4	Bristol/1993/UK	X76716	Lordsdale, Camberwell, Grimsby, New Orleans, Sydney
	5	Hillingdon/1990/UK	AJ277607	White river
	6	Seacroft/1990/UK	AJ277620	
	7	Leeds/1990/UK	AJ277608	Gwynedd, Venlo, Creche
	8	Amsterdam/1998/NL	AF195848	
	9	VA97207/1997/US	AY038599	
	10	Erfurt546/2000/DE	AF427118	
	11	Sw918/1997/JP	AB074893	
	12	Wortley/1990/UK	AJ277618	
	13	Fayetteville/1998/US	AY113106	
	14	M7/1999/US	AY130761	
	15	J23/1999/US	AY130762	
	16	Tiffin/1999/US	AY502010	
	17	CS-E1/2002/US	AY502009	
	18	OH-QW101/2003/US	AY82330	
	19	OH-QW170/2003/US	AY823306	

(continued)

Table 3.1 (continued)

Geno-group	*Geno-type*	*Prototype strain*[a]	*GenBank accession number (capsid gene)*	*Other strains*
	20	Luckenwalde591/2002/DE	EU373815	
	21	IF1998/2002/IQ	AY675554	
	22	Yuri/2003/JP	AB083780	
III	1	Jena/1980/DE	AJ011099	Bovine strains
IV	1	Alphatron/1998/NL	AF195847	Ft. Lauderdale
V	1	MNV-1/2002/US	AY228235	Murine strains
VI	1	Bari91/2007/IT	FJ875027	

[a]*CA* Canada, *DE* Germany, *IQ* Iraq, *IT* Italy, *JP* Japan, *NL* Netherlands, *SA* Saudi Arabia, *UK* United Kingdom, *US* United States

sequences to fully describe the genotypic features of clinical strains (Buesa et al. 2002; Bull et al. 2007; Hoa Tran et al. 2013). Recombination is a potentially important means by which these viruses generate diversity (Bull et al. 2007; Eden et al. 2013). Dual genotyping based on the polymerase encoding region and the capsid amino acid sequence can be conducted by using a web-based tool: http://www.rivm.nl/mpf/norovirus/typingtool (Kroneman et al. 2011). The method employs percent pairwise similarities in reference to prototype strains. Thus, the polymerase genotype is defined with a cut-off value of ≥15 % for the polymerase nucleotide sequence (about 800 nt at the 3′ end of ORF1), whereas the capsid genotype is defined with a cut-off value of ≥15 % for the major capsid protein (VP1) amino acid sequence (Vinjé et al. 2004; Zheng et al. 2006). Thus far, 14 GI and 29 GII polymerase genotypes and 9 GI and 22 GII capsid genotypes have been described (RIVM-NoroNet) (Kroneman et al. 2013). Genotype GII.4 is further divided into variants (subgenotypes) based on sequence diversity (Vinjé et al. 2004; Siebenga et al. 2007; Buesa et al. 2008; Kroneman et al. 2011). New GII.4 variants are recognized only after evidence is provided that they have become epidemic lineages in at least two geographically diverse locations. Table 3.2 shows the new consensus nomenclature for epidemic norovirus GII.4 variants (Kroneman et al. 2013).

Molecular characterization of bovine enteric caliciviruses suggests their inclusion into GIII, which contains viruses so far found only in cattle (Ando et al. 2000; Oliver et al. 2003; Smiley et al. 2003). Phylogenetic analysis places at least two human noroviruses within genogroup IV: strains Alphatron (GenBank accession number AF195847) and Ft. Lauderdale (GenBank accession number AF414426) (Fankhauser et al. 2002). The porcine noroviruses cluster within GII (Sugieda and Nakajima 2002). The murine noroviruses have been included into GV (GenBank accession number DQ285629), members of which are closer to GII than those of GI by sequence alignment (Karst et al. 2003). In the

Table 3.2 Proposed epidemic norovirus GII.4 variants (adapted from Kroneman et al. (2013) and Kroneman et al. (2011))

Proposed epidemic variant name	*GenBank no.*[a]
US95_96	AJ004864[b]
Farmington_Hills_2002	AY485642[c]
Asia_2003[d]	AB220921[c]
Hunter_2004	AY883096[b]
Yerseke_2006a	EF126963[b]
Den Haag_2006b	EF126965[b]
NewOrleans_2009	GU445325[c]
Sydney_2012[e]	JX459908[c]

[a]GenBank accession number of the first submitted capsid sequence of this variant
[b]Capsid sequence
[c]Complete genome
[d]Variant Asia_2003 is a recombinant with a GII.4 ORF2 and a GII.P12 ORF1
[e]Variant Sydney_2012 is a recombinant with a GII.4 ORF2 and a GII.Pe ORF1

major capsid protein VP1, human norovirus strains within the same genogroup share at least 60 % amino acid sequence identity, whereas most GI strains share less than 50 % amino acid identity with GII strains (Green et al. 2001; Koopmans et al. 2003). Picornavirus serotypes generally have >85 % amino acid identity across the VP1 gene, which is in the range of the cut-off for calicivirus genotypes (>80 % amino acid identity) (Oberste et al. 1999).

Caliciviruses contain subgenomic RNA encoding regions that span from the start of the capsid gene to the 3′ end (Fig. 3.1). It has been hypothesized that the subgenomic RNA could act as an independent unit participating in recombination events. If RNA recombination is a common phenomenon among caliciviruses, high diversity of the family might be expected, which would facilitate the emergence of new variants and make genotyping more difficult (Bull et al. 2012). Recombination may permit caliciviruses to escape host immunity, analogous to antigenic shifts by influenza viruses, but by a different molecular mechanism. It has also been reported that accumulation of mutations in the protruding P2 domain of the capsid protein may result in predicted structural changes, including disappearance of a helix structure of the protein, and thus a possible emergence of new phenotypes (Nilsson et al. 2003; Lindesmith et al. 2011).

1.4. Genetic Classification of Sapoviruses

The sapovirus major capsid protein-encoding region is fused to and in frame with the polyprotein encoding region (Lambden et al. 1994; Numata et al. 1997). All published sapovirus strains except a human strain, London/92, and

Table 3.3 Classification of current genogroups and genotypes of sapoviruses (adapted from Schuffenecker et al. (2001) and Farkas et al. (2004))

Genogroup/ cluster	*Prototype strain*	*GenBank accession number (capsid gene)*	*Other strains*
GI/1	Sapporo/82	U65427	Houston/86, Plymouth/92, Manchester/93, Lyon30388/98
GI/2	Parkville/94	U73124	Houston/90
GI/3	Stockholm/97	AF194182	Mexico14917/00
GII/1	London/92	U95645	Lyon/598/97
GII/2	Mexico340/90	AF435812	
GII/3	Cruise ship/00	AY289804	
GIII	PEC/Cowden	AF182760	
GIV	Houston7-1181/90	AF435814	
GV	Argentina39	AY289803	

a porcine strain, PEC Cowden, contain an additional ORF predicted in +1 frame, overlapping the N terminus of the capsid encoding region (Guo et al. 1999b; Jiang et al. 1999; Clarke and Lambden 2001). By analogy with the classification of the noroviruses, the sapoviruses were previously divided into four genotypes, belonging to two genogroups (Liu et al. 1995; Noel et al. 1997; Clarke and Lambden 2000). More recently, human sapoviruses have been classified into five genogroups (Schuffenecker et al. 2001; Farkas et al. 2004) (Table 3.3). In addition, the porcine enteric calicivirus (Cowden strain) has been shown to be related to the *Sapovirus* genus but belongs to a differentiated cluster (Guo et al. 1999b). Further classification of sapovirus into genotypes has also been undertaken, though taxonomic assignment at the genotype level appears to be less well-defined than at the genogroup level (L'Homme et al. 2010; Oka et al. 2012). Members of SaV GI, GII, GIV, and GV are found to infect humans and those of GIII are found in swine (Farkas et al. 2004). Each genogroup can be further subdivided into a number of genotypes or genetic clusters. Recent studies underscore the ambiguity in classification of animal SaVs beyond the species level and serves as a caution against proposing novel *Sapovirus* genogroups based only on short polymerase sequences (L'Homme et al. 2009a).

1.5. Virus Replication

Studies on the replication strategy of human caliciviruses have been hampered by the lack of an efficient cell culture system or an appropriate animal model. Nevertheless, the first mouse model for HuNoVs has recently been reported; it consists of BALB/c mice deficient in recombination activation gene (Rag) 1 or 2 and common gamma chain (γc) ($Rag^{-/-}\gamma c^{-/-}$ mice) (Taube et al. 2013). If validated, this model has the potential to accelerate our

knowledge of human norovirus biology and help investigate the molecular mechanisms regulating norovirus infections.

Expression of recombinant proteins from cDNA clones has allowed the generation of proteolytic processing maps for the nonstructural proteins of several caliciviruses e.g., Southampton virus (a norovirus) and RHDV (a lagovirus) (Liu et al. 1996; Wirblich et al. 1996). Analysis of individual recombinant proteins from these noncultivable caliciviruses allowed the identification of NTPase and 3C-like cysteine protease activities for RHDV and noroviruses (Liu et al. 1999) and a 3D-like RNA-dependent RNA polymerase for RHDV (López-Vazquez et al. 1998). Studies on the replication mechanisms of cultivatable caliciviruses, like feline calicivirus (FCV), have contributed to a better understanding of the basic features of calicivirus replication (Sosnovtsev and Green 2003). The FCV replicates by producing two major types of polyadenylated RNAs; a positive-sense genomic RNA of approximately 7.7 kb, and a subgenomic RNA of 2.4 kb (Herbert et al. 1996b). The genomic RNA serves as a template for synthesis of the nonstructural protein encoded by the ORF1, whereas the subgenomic RNA is a template for translation of structural proteins (Carter 1990).

Guix et al. (2007) demonstrated that transfection of Norwalk virus RNA into human hepatoma Huh-7 cells leads to viral replication with expression of viral antigens, RNA replication, and release of viral particles into the medium. Prior treatment of RNA with proteinase K completely abolished RNA infectivity, suggesting a key role of a RNA-protein complex. However, a block to viral spread to other cells in the culture remained, indicating that the blockade exists at the stage of cell entry and/or uncoating (Guix et al. 2007). A number of host factors are important in norovirus life cycle as demonstrated by proteomics and reverse genetics (Bailey et al. 2010; Yunus et al. 2010). It has been shown that the norovirus VPg protein interacts with the host cell cap-binding proteins eIF4E and eIF4G (Chaudhry et al. 2007). The crystallographic structures of the VPg proteins from FCV and MNV have been recently determined by nuclear magnetic resonance spectroscopy (Leen et al. 2013).

Reverse genetics and replicon systems have become important tools to elucidate the mechanism of calicivirus replication and pathogenicity. Reverse genetics systems are available for cultivatable viruses such as FCV (Sosnovtsev and Green 1995), MNV (Ward et al. 2007), porcine enteric calicivirus (Chang et al. 2005), RHDV (Liu et al. 2008) and Tulane virus, a rhesus monkey calicivirus (Wei et al. 2008). For uncultivatable caliciviruses, such as human norovirus, replicon systems with either transient or stable expression of viral RNA have been developed. Transfection of a full-length cDNA clone of the Norwalk virus RNA under the control of T7 promoter into MVA-T7 infected cells allowed the expression of viral proteins and Norwalk virus RNA replication (Asanaka et al. 2005). A cell-based replicon was generated for Norwalk virus replication using a cloned cDNA consensus sequence of the Norwalk virus genome (Fernandez-Vega et al. 2004) engineered to encode the neomycin resistance gene as a selective marker within ORF2. However, only few

transfected cells could apparently support virus replication, suggesting that severe growth restrictions were present in the cells (Chang et al. 2006; Chang et al. 2008).

1.6. Virus-Cell Interactions

Human and animal enteric caliciviruses are assumed to replicate in the upper intestinal tract, causing cytolytic infections in the villous enterocytes but not in the crypt enterocytes of the proximal small intestine. Biopsies of the jejunum taken from experimentally infected volunteers who developed gastrointestinal disease following oral administration of noroviruses showed histopathologic lesions consisting of: blunting of the villi, crypt cell hyperplasia, infiltration with mononuclear cells, and cytoplasmic vacuolization (Blacklow et al. 1972; Dolin et al. 1975). Experiments with recombinant Norwalk VLPs and human gastrointestinal biopsies showed that VLPs bind to epithelial cells of the pyloric region of the stomach and to enterocytes on duodenal villi. Attachment of the rVLPs occurred only on cells as well as to saliva from histo-blood group antigen (HBGA)-secreting individuals (Marionneau et al. 2002). It was previously determined that RHDV attaches to H type 2 HBGAs present on rabbit epithelial cells (Ruvöen-Clouet et al. 2000). Significant attachment of Norwalk rVLPs onto differentiated Caco-2 cells has been demonstrated (White et al. 1996). Differentiated Caco-2 cells, derived from an individual expressing the group O blood type, resemble mature enterocytes and express H-type HBGAs on their cell surfaces (Amano and Oshima 1999).

To date, the Cowden strain of porcine enteric calicivirus (PEC) is the only cultivatable enteric sapovirus (Flynn and Saif 1988). However, for replication, it requires the incorporation of an intestinal contents preparation (ICP) from uninfected gnotobiotic pigs or bile acids as a medium supplement (Chang et al. 2004). Different porcine intestinal enzymes such as trypsin, pancreatin, alkaline phosphatase, enterokinase, elastase, protease and lipase were tested as medium supplements, but none of them alone promoted viral growth in cell culture (Parwani et al. 1991). It was speculated that some enzymes or factors in the porcine ICP could activate the viral receptor, promote signalling of host cells, or might help in cleavage of the viral capsid for successful uncoating (Guo and Saif 2003).

Although noroviruses are highly infectious (it has been estimated that as low as 18 and as high as 2800 virions are enough to infect an adult (Atmar et al. 2014; Teunis et al. 2008) studies in volunteers have shown that some subjects remain uninfected despite having been challenged with a high dose of virus (Matsui and Greenberg 2000). This could be due to the presence of innate resistance or pre-existing immunity to the virus (Lindesmith et al. 2003). An increased risk of Norwalk virus infection has been associated with blood group O; and Norwalk VLPs bind to gastroduodenal cells from individuals who are secretors (Se+) but not to those from non-secretors (Se-) (Hutson et al. 2002; Marionneau et al. 2002). The gene responsible for the

secretor phenotype *FUT2* encodes an α(1,2) fucosyltransferase that produces H type HBGAs found on the surface of gastrointestinal epithelial cells and in mucosal secretions (Lindesmith et al. 2003). Additional forms of HBGAs found on the gut mucosa and its secretions depend on additional glycosyltransferases, including those that produce the Lewis antigens and the A- and B-type antigens related to those present on red blood cells (Marionneau et al. 2001). The discovery that noroviruses attach to cells in the gut only if the individual expresses specific, genetically-determined HBGA carbohydrates is a breakthrough in understanding norovirus-host interactions and susceptibility to norovirus infections (Lindesmith et al. 2003; Hutson et al. 2004). Individuals with defects in the *FUT2* gene are termed secretor-negative, do not express the appropriate HBGAs necessary for Norwalk virus binding, and are therefore resistant to Norwalk virus infection. These data argue that *FUT2* and other genes encoding enzymes that regulate the processing of the HBGA carbohydrates function as susceptibility alleles. However, secretor-negative individuals can be infected with other norovirus strains (Le Pendu et al. 2006; Lindesmith et al. 2008).

To explore the process of norovirus attachment to cells, virus-like particles (VLPs), which mimic native virions morphologically and antigenically, have been used (White et al. 1996; Lindesmith et al. 2003; Allen et al. 2008). The C-terminal of the P-domain of NoVs has the ability to auto-assemble into subviral particles, termed P particles (Tan and Jiang 2005), which are potentially useful for broad applications including vaccine development (Tan et al. 2011). The C-terminal region (P domain) of the capsid protein is involved in HBGA attachment and, therefore, plays an essential role in cell infection (Marionneau et al. 2002; Hutson et al. 2003). The crystal structures of the HBGA-binding interfaces of Norwalk virus (GI.1) and VA387 (GII.4) have been elucidated, each representing one of the two major genogroups of human noroviruses (Cao et al. 2007; Choi et al. 2008). The HBGA-binding interfaces of the two strains differ significantly in their structures, precise locations of HBGA-binding locations and amino acid compositions, although both locate on the top of the arch-like P dimer of the viral capsids (Tan et al. 2009). Sequence alignment has shown that the key residues responsible for HBGA binding are highly conserved within but not between genogroups GI and GII and the remaining sequences of the P2 subdomain are highly variable (Tan et al. 2009; Tan and Jiang 2010). It has been suggested that noroviruses may use secondary or alternative receptors other than HBGAs when attaching to enterocytes. Hence HBGAs could play a role as co-receptors but blocking such interactions may not abolish attachment to these cells (Murakami et al. 2013).

Recent studies also suggest that some animal caliciviruses may cross the species barrier and potentially infect humans. The hypothetical existence of animal reservoirs and the possibility of interspecies transmission have been suggested by phylogenetic linkage between human and bovine or porcine viruses within the genera *Norovirus* and *Sapovirus*, respectively (Clarke and

Lambden 1997; Dastjerdi et al. 1999; Liu et al. 1999; van Der Poel et al. 2000). However, information concerning the frequency of interspecies transmission among caliciviruses is limited. Given the genetic similarity of human and animal caliciviruses and their potential for recombination, interspecies transmission of noroviruses and sapoviruses is possible, although not demonstrated to date.

2. ROTAVIRUSES

Rotaviruses are the leading cause of viral gastroenteritis in infants and young children worldwide and also in the young of a large variety of animal species (Parashar et al. 2003; Parashar et al. 2006). Rotavirus infections in humans continue to occur throughout their lives but the resulting disease is mild and often asymptomatic (Bishop 1996). In addition to sporadic cases of acute gastroenteritis, outbreaks of rotavirus diarrhea in school-aged children and adults have increasingly been reported (Griffin et al. 2002; Mikami et al. 2004; Rubilar-Abreu et al. 2005). Rotaviruses are known to produce disease in humans since 1973 (Bishop et al. 1973; Flewett et al. 1973a). They are responsible for an estimated 500,000 deaths each year, mostly in infants and young children in developing countries (Parashar et al. 2009). The main strategy to combat rotavirus infection has been the development of rotavirus vaccines. Since the 1980s this has been the focus of much rotavirus research and since 2006, two vaccines have been licensed in many countries around the world.

2.1. Virus Classification

The classical classification system for rotaviruses was derived from their genome composition and the immunological reactivity of three of their structural proteins VP6, VP7 and VP4. Rotaviruses are classified into at least eight groups (A to H) according to the immunological reactivity of the VP6 middle layer protein. Group A rotaviruses are commonly associated with infections in humans. Within group A, two major subgroups (I and II) exist (Iturriza Gomara et al. 2002). The two outer capsid proteins, VP7 and VP4, elicit neutralizing antibodies and are considered to be involved in protection against infection. Using these two proteins a traditional dual classification system of group A rotaviruses into G (depending on VP7 that is a Glycoprotein) and P (from the VP4 that is sensitive to Proteases) types was established (Estes and Kapikian 2007). At least 27 different G-serotypes and 37 P-types have been identified among human and animal rotaviruses, depending on VP7 and VP4, respectively. G serotypes correlate fully with G genotypes as determined by sequence analysis of their VP7 gene. However, many P genotypes do not correlate with P serotypes (Estes and Kapikian 2007). Because VP4 and VP7 are coded by different RNA segments (segment 4 and segments 7–9, respectively), various combinations of G- and P-types can be found in both humans and animals. Viruses

carrying G1P[8], G2[P4], G3[P8] and G4[P8] represent over 90 % of human rotavirus strains co-circulating in most countries, although other G and P combinations are also being isolated in increasing numbers (Iturriza-Gomara et al. 2011). Besides this dual classification system, another system has been utilized including the genotype of the rotavirus enterotoxin, the NSP4 gene (Rodriguez-Diaz et al. 2008; Banyai et al. 2009).

More recently a classification system including the whole genome has been established by the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al. 2008b; Matthijnssens et al. 2011). This classification system allows a better understanding of genomic and antigenic diversity of rotaviruses as well as the reassortment events present in the rotavirus strains. In this genomic classification, each of the 11 RNA segments has been given a tag that includes the traditional G for VP7, P for VP4 protein and E for the NSP4 protein (NSP4 is the viral enterotoxin). The complete genome classification code is as follows: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx correlating with the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 encoding genes, respectively (Matthijnssens et al. 2008b; Matthijnssens et al. 2011). Whole genome analysis of rotavirus strains is generating interest in their complete genetic constellations and complex genetic diversity (Matthijnssens and Van Ranst 2012). On the basis of complete group A rotavirus genome sequence comparisons, two major genotype constellations of the non-G, non-P genes; I1-R1-C1-M1-A1-N1-T1-E1-H1 (Wa-like) and I2-R2-C2-M2-A2--N2-T2-E2-H2 (DS-1-like), have been shown to circulate worldwide among humans. Human Wa-like rotavirus A (RVA) strains are believed to have a common ancestor with porcine RVA strains, whereas human DS-1-like strains are believed to have several gene segments which have a common ancestor with bovine RVA strains (Matthijnssens et al. 2008a). A third (minor) human genotype constellation, referred to as AU-1-like (I3-R3-C3-M3-A3-N3-T3-E3-H3), is believed to originate from cats or dogs (Nakagomi et al. 1990).

2.2. Structure of the Virion

The wheel-like structure is the characteristic feature of RV particles under transmission electron microscopy and has the name; “rota” in Latin means wheel. Rotavirus particles are of icosahedral symmetry, consist of three concentric layers of protein and measure ~1000 Å in diameter including the spikes (Estes and Kapikian 2007). The core layer is formed by the VP2 protein entirely surrounding the 11 segments of double-stranded genomic RNA, and proteins VP1 and VP3, which are transcription enzymes attached as a heterodimeric complex to the inside surface of VP2 at the fivefold axis of symmetry (Prasad et al. 1996b) (Fig. 3.3). VP1, the RNA-dependent RNA polymerase, interacts with VP3, the guanyltransferase and methylase protein (Liu et al. 1992). This inner core structure is composed of 120 copies of VP2, which is a RNA-binding protein (Labbe et al. 1991). The addition of VP6 to the VP2 layer produces double-layered particles (DLP). VP6 forms 260 trimers interrupted by 132 aqueous channels of three different kinds in relation

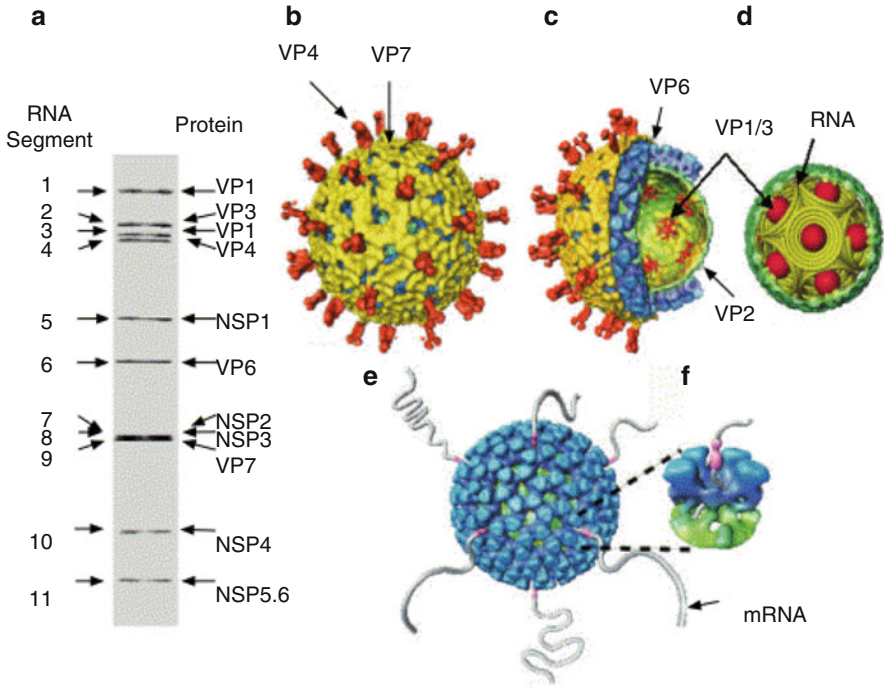

Figure 3.3. Architectural features of rotavirus. (**a**) PAGE gel showing 11 dsRNA segments composing the rotavirus genome. The gene segments are numbered on the *left* and the proteins they encode are indicated on the *right*. (**b**) Cryo-EM reconstruction of the rotavirus triple-layered particle. The spike proteins VP4 are colored in orange and the outermost VP7 layer in yellow. (**c**) A cutaway view of the rotavirus TLP showing the inner VP6 (*blue*) and VP2 (*green*) layers and the transcriptional enzymes (shown in *red*) anchored to the VP2 layer at the fivefold axes. (**d**) Schematic depiction of genome organization in rotavirus. The genome segments are represented as inverted conical spirals surrounding the transcription enzymes (shown as *red balls*) inside the VP2 layer in *green*. (**e** and **f**) Model from cryo-EM reconstruction of transcribing DLPs. The endogenous transcription results in the simultaneous release of the transcribed mRNA from channels located at the fivefold vertex of the icosahedral DLP (Reproduced with permission from Jayaram et al. 2004).

to the capsid's symmetry. The outer capsid of the triple-layered particles (TLP) is composed of two proteins, VP7 and VP4. The smooth surface of the virus is made up of 260 trimers of VP7 and 60 spikes emerging from the viral surface consist of dimers of VP4 (Prasad et al. 1988; Yeager et al. 1990). Rotavirus DLP and TLP contain 132 porous channels which allow the exchange of compounds to the inside of the virus particle. There are 12 type I channels each of which is located at the icosahedral fivefold vertices of the TLP and DLP. Each of the type I channels is surrounded by five type II channels, and 60 type III channels are placed at the hexavalent positions

immediately neighboring the icosahedral threefold axis (Prasad et al. 1988; Yeager et al. 1990).

During cell entry, the TLP loses the VP7 and VP4 proteins, and the resulting DLP becomes transcriptionally active inside the cytoplasm (Estes and Kapikian 2007). VP4 is a non-glycosylated protein of 776 amino acids and has essential functions in the virus cell cycle, including receptor binding, cell penetration, hemagglutinin activity, and permeability of cellular membranes (Estes and Kapikian 2007). VP4 is post-transcriptionally cleaved into the larger VP5* and the smaller VP8* subunits; the cleavage of VP4 enhances virus infectivity by several fold. It has been shown that trypsin cleavage confers icosahedrical ordering on the VP4 spikes, which is essential for the virus to enter the cell (Crawford et al. 2001). Moreover, biochemical studies on recombinant VP4 showed that proteolysis of monomeric VP4 yields dimeric VP5* (Dormitzer et al. 2001). VP7 is a calcium-binding glycoprotein of 326 amino acids with nine variable regions contributing to type-specificity (Nishikawa et al. 1989; Hoshino et al. 1994). In addition, VP7 interacts with integrins αxβ2 and α4β1 (Coulson et al. 1997a; Hewish et al. 2000) and induces polyclonal intestinal B-cell activation during rotavirus infection (Blutt et al. 2004).

When the DLP is located intracellularly, it becomes transcriptionally competent and new mRNA transcripts are translocated from the particle through the type I channels at the fivefold axis (Lawton et al. 1997). Prasad et al. (1988) were the first to propose that these channels in the VP6 layer could be used by mRNA to exit. Cryo-EM studies have confirmed that DLPs maintain their structural integrity during the process of transcription. In a pseudo-atomic model of the T = 13 VP6 layer (Mathieu et al. 2001), a β-hairpin motif of VP6 with a highly conserved sequence that protrudes into the mRNA exit channel may play a functional role in the translocation of the nascent transcripts (Lawton et al. 2000). A detailed mutational analysis of the VP6 layer has contributed to elucidation of determinants of VP6 required for its assembly on VP2 and how VP6 may affect endogenous transcription (Charpilienne et al. 2002).

2.3. The Genome

The genome of rotavirus consists of 11 segments of double-stranded RNA (dsRNA) with conserved 5′ and 3′ ends, ranging from 667 bp (segment 11) to 3302 bp (segment 1) in size. The simian rotavirus strain SA11 totals 6120 kDa and its genes encode for six structural proteins (VP1, VP2, VP3, VP4, VP6, VP7) and six nonstructural proteins (NSP1 to NSP6). The coding assignments, functions and many properties of the proteins encoded by each of the 11 genome segments are now well established (Table 3.4). Protein assignments have been determined by in vitro translation using mRNA or denatured dsRNA and by analyses of reassortant viruses (Estes and Kapikian 2007).

Cryo-EM analysis of rotavirus provided visualization of the structural organization of the viral genome (Prasad et al. 1996b). It was shown that the dsRNA forms a dodecahedral structure in which the RNA double helices,

Table 3.4 Genome segments and proteins of simian rotavirus SA11: coding assignments, functions and biological properties of the encoded proteins (adapted from Estes (2001) and Estes and Kapikian (2007))

Genome segment size (bp)	*ORFs*	*Gene product(s)*	*Protein size aa (Da)*	*Location in virus particle*	*Copy number/ Particle*	*Functions and properties*
1 (3302)	18-3282	VP1	1088 (125,005)	Inner capsid, fivefold axis	12	RNA-dependent RNA polymerase Part of minimal replication comlex Virus specific 3′-mRNA binding Part of virion transcription complex with VP3
2 (2690)	17-2659	VP2	881 (102,431)	Inner capsid	120	Core matrix protein Non-specific ss & dsRNA-binding activity Myristoylated Assembly and RNA-binding activity Part of minimal replication complex Leucine zipper
3 (2591)	50-2554	VP3	835 (98,120)	Inner capsid, fivefold axis	12	Multifunctional capping enzyme: Guanylyltransferase Methyltransferase Located at the vertices of the core Part of virion transcription complex with VP1 Non-specific ssRNA binding
4 (2362)	10-2337	VP4	776 (86,782)	Outer capsid spike	120	VP4 dimers form outer capsid spike Interacts with VP6
	–	VP5*	529 247–776 (60,000)			Virus infectivity enhanced by trypsin cleavage of VP4 into VP5* and VP8* Hemagglutinin Cell attachment protein
	–	VP8*	247 1–247 (28,000)			P-type neutralization antigen VP5* permeabilizes membranes Crystal structure of VP8 fragment (galectin fold) TRAF2 signaling Protection

5 (1611)	31-1515	NSP1	495 (58,654)	Nonstructural	–	Associates with cytoskeleton Extensive sequence diversity between strains Two conserved cysteine-rich zinc-finger motifs Virus specific 5′-mRNA binding Interacts with host IFN regulatory factor 3
6 (1356)	24-1214	VP6	397 (4816)	Middle capsid	780	Major virion protein Middle capsid structural protein Homotrimeric structure Subgroup antigen Myristoylated [15]. Protection (Mechanism?)
7 (1105)	26-970	NSP3	315 (34,600)	Nonstructural	–	Homodimer Virus-specific 3′- mRNA binding Binds eIF4G1 and circularizes mRNA on initiation complex Involved in translational regulation and host shut-off
8 (1059)	47-997	NSP2	317 (36,700)	Nonstructural	–	NTPase activity Helix destabilization activity Non-specific ssRNA-binding Involved in viroplasm formation with NSP5 Functional octamer Binds NSP5 and VP1 Induces NSP5 hyperphosphorylation
9 (1062)	49-1026	VP7	326 (7368)	Outer capsid glycoprotein	780	Outer capsid structural glycoprotein G-type neutralization antigen N-linked high mannose glycosylation and trimming RER transmembrane protein, cleaved signal sequence Ca^{2+} binding Protection

(continued)

Table 3.4 (continued)

Genome segment size (bp)	*ORFs*	*Gene product(s)*	*Protein size aa (Da)*	*Location in virus particle*	*Copy number/ Particle*	*Functions and properties*
10 (751)	41-569	NSP4	175 (20,290)	Nonstructural	–	Enterotoxin Receptor for budding of double-layer particle through ER membrane RER transmembrane glycoprotein Ca++/ Sr++ binding site N-linked high mannose glycosylation Protection Host cell $[Ca^{2+}]_i$ mobilization Pathogen-associated molecular pattern (PAMP)
11 (667)	22-615	NSP5	198 (21,725)	Nonstructural	–	Interacts with VP2, NSP2 and NSP6 Homomultimerizes O-linked glycosylation (Hyper-) Phosphorylated Autocatalytic kinase activity enhanced by NSP2 interaction Non-specific ssRNA binding
	80-355	NSP6	92 (11,012)	Nonstructural	–	Product of second, out-of-frame ORF Interacts with NSP5 Localizes to viroplasm

interacting closely with the VP2 inner layer, are packed around the transcription complexes located at the icosahedral vertices (Fig. 3.3d). VP2 has RNA binding properties and may be responsible for the icosahedral ordering and the closely interacting portions of the RNA. Pesavento et al. (2001) have demonstrated reversible condensation and expansion of the rotavirus genome within the capsid interior under various chemical conditions. This condensation is concentric with respect to the particle center and a dark mass of density is consistently seen in the center of each of the particles (Pesavento et al. 2001). At high pH in the presence of ammonium ions, the genome condenses to a radius of 180 Å from the original radius of 220 Å, and when brought back to physiological pH the genome expands to its original radius (Pesavento et al. 2001). These studies suggest that VP2, through its RNA-binding properties, plays an important role in maintaining the appropriate spacing between the RNA strands in the native expanded state. A plausible and elegant model for the structural organization of the genome that emerges from these studies is that each dsRNA segment is spooled around a transcription enzyme complex at the fivefold axes, inside the innermost capsid layer (Prasad et al. 1996b; Pesavento et al. 2001; Pesavento et al. 2003).

2.4. Mechanisms of Evolution and Strain Diversity

Rotaviruses, like many RNA viruses, display a great degree of diversity. In addition to different G and P types and a variety of combinations of these, there also exists intratypic variation. Three mechanisms, singularly or in combination, are important for the evolution and diversity of rotaviruses, although it is not clear what their relative contributions are to the burden of disease:

(a) *Antigenic drift:* The rate of mutation within rotavirus genes is relatively high because RNA replication is error-prone. The mutation rate has been calculated to be $<5 \times 10^{-5}$ per nucleotide per replication which implies that on average, a rotavirus genome differs from its parental genome by at least one mutation (Blackhall et al. 1996). Point mutations can accumulate and give rise to intratypic variation as identified by the existence of lineages within the VP7 and VP4 genes of particular G and P types. Point mutations can lead to antigenic changes, which may result in the emergence of escape mutants (Palombo et al. 1993; Cunliffe et al. 1997; Maunula and von Bonsdorff 1998; Iturriza Gomara et al. 2002; Estes and Kapikian 2007).

(b) *Antigenic shift:* Shuffling of gene segments through reassortment can occur during dual infection of one cell. Reassortment can, therefore, contribute to the diversity of rotaviruses and there is increasing evidence that reassortment takes place *in vivo* (Ramig 2000). There is also evidence that reassortment, through alterations in protein-protein interactions possibly leading to changes in conformational epitopes, can contribute to the evolution of antigenic types (Chen et al. 1992; Lazdins et al. 1995). Interspecies transmission and the subsequent reassortment have the potential to

enormously increase the diversity of co-circulating rotaviruses. In addition, human rotavirus genes encoding proteins to which the human population is immunologically naïve may allow a rapid spread of the reassortant strain (Iturriza-Gomara et al. 2000). While antigenic drift has always been considered one of the main forces driving rotavirus evolution, extensive analysis of whole genome sequences seems to reveal there are restrictions to this evolution mechanism (Matthijnssens and Van Ranst 2012).

(c) *Gene rearrangements:* The concatemerization or truncation of genome segments and their ORFs has the potential to contribute to the evolution of rotaviruses through the production of new proteins with altered functions (Desselberger 1996).

2.5. Genome Replication

The RNA polymerase activity of DLPs catalyzes the synthesis of the 11 mRNAs of rotaviruses, which range in size from ~0.7 to 3.3 kb. With the exception of gene 11, they are monocistronic (Estes and Kapikian 2007). The nascent transcripts are extruded through channels present at the fivefold-axes of the DLPs (Lawton et al. 1997). The 5′- and 3′-untranslated regions (UTRs) are 9–49 and 17–182 nucleotides in length, respectively. The viral mRNAs serve as templates for the synthesis of minus-strand RNA, to form dsRNA molecules (Chen et al. 1994). The synthesis of dsRNA and the assembly of cores and DLPs occur in viroplasms in the cytoplasm (Estes and Kapikian 2007). There are three species of RNA-containing replication intermediates (RIs) in the infected cells: pre-core RIs, which contain the structural proteins VP1 and VP3; core-RIs with VP1, VP2 and VP3; and double layered RIs, which contain VP1, VP2, VP3 and VP6. The 11 genomic segments are produced and packaged in equimolar amounts in rotavirus-infected cells, which demonstrates that RNA packaging and replication are coordinated processes (Patton and Gallegos 1990). The absence of naked dsRNA in infected cells suggests that packaging takes place before replication (Patton et al. 2003).

The only primary sequences that are conserved among the rotavirus mRNAs are located within the UTRs. Since all 11 mRNAs are replicated by the same VP1-VP2-VP3 polymerase complex, the viral mRNAs almost certainly share common cis-acting signals recognized by the polymerase (Patton et al. 2003). The most remarkable feature of the 3′ end of rotavirus mRNA, as well as that of other members of the *Reoviridae* family, is the absence of a poly(A) tail. Instead, all rotavirus genes and mRNAs end with the same short sequence, UGACC, which is conserved amongst all the segments in group A rotaviruses. Site-specific mutagenesis has revealed that it is the 3′-CC of the 3′ consensus sequence that is most critical for minus-strand synthesis (Chen et al. 2001). In addition, it has been demonstrated that the promoter for minus-strand synthesis is formed by base-pairing in *cis* of complementary sequences proximal to the 5′- and 3′-ends of the viral mRNAs (Chen and Patton 1998). The 3′-consensus sequence also contains a cis-acting signal that acts as a translation enhancer, whose activity is mediated by NSP3 that specifically recognizes the last four to five nucleotides of the 3′-consensus sequence (Poncet

et al. 1994). NSP3 also interacts with the initiation factor, eIF4GI, and facilitates the circularization of viral mRNAs in polysomes, hence increasing the efficiency of viral gene expression (Piron et al. 1998).

The development of a cell-free system that supports the synthesis of dsRNA from exogenous mRNA represents an important milestone in the study of rotavirus replication by providing a tool to analyze the elements in viral mRNAs that promote minus-strand synthesis. This system is based on virion-derived cores which have been disrupted or "opened" by incubation at hypotonic buffer (Chen et al. 1994). Recently, two systems that allow partial reverse genetics on rotavirus have emerged. The first approach utilized a helper virus and neutralizing selection antibodies (Komoto et al. 2006; Komoto et al. 2008). However, in this approach only structural proteins that are able to elicit specific neutralizing antibodies could be modified. In the second approach, the combination of a thermosensitive defect in the NSP2 protein with RNAi-mediated degradation of NSP2 mRNAs allowed the dual selection and recovery of recombinant viruses with modifications in a non-structural protein coding gene (Trask et al. 2010; Navarro et al. 2013).

2.6. Cell Infection

Rotaviruses have a specific tropism *in vivo*, infecting primarily the mature enterocytes of the villi of the small intestine. Several reports suggest that extra-intestinal spread of the virus takes place during infection indicating a wider tropism than previously considered (Blutt et al. 2003; Mossel and Ramig 2003). Rotaviruses can bind to a wide variety of cell lines although only a subset is efficiently infected. These include cells of renal and intestinal origin and transformed cell lines from breast, stomach, lung and bone (Ciarlet et al. 2002b; Lopez and Arias 2004). Most studies on rotavirus replication have been carried out in MA104 cells, which are routinely used to produce progeny virus. However, new investigations into the pathophysiological mechanisms of rotavirus infection are now being performed using in vitro polarized cells like the human intestinal HT-29 and Caco-2 cells (Servin 2003).

Rotaviruses enter into the cell by a complex multistep process in which different domains of the viral surface interact with cell surface molecules that act as receptors (Guerrero et al. 2000a; Guerrero et al. 2000b; Ciarlet and Estes 2001; Ciarlet et al. 2002a; Ciarlet et al. 2002b; Lopez and Arias 2004). Some animal rotavirus strains interact with sialic acid (SA) residues to attach to the cell surface and hence their infectivity is diminished by the treatment of cells with neuraminidase (NA). In contrast, many animal and human strains are NA-resistant (Ciarlet and Estes 1999). The interaction of rotavirus with SA has been shown to depend on the VP4 genotype of the virus and not the species of origin (Ciarlet et al. 2002b). Ganglioside GM3 has been suggested to act as the SA-containing receptor for the porcine rotavirus strain OSU (Rahman et al. 2003) and ganglioside GM1 (NA-resistant) has been identified as the receptor for the NA-resistant human rotavirus strains KUN and MO (Guo et al. 1999a).

The VP8* domain of VP4 is involved in interactions with SA whereas VP5* is implicated in interactions with integrins. The interaction of rotavirus with integrin α2β1 has been shown to be mediated by the DGE integrin-recognition motif, located at amino acids 308–310 of VP4 within VP5* (Zárate et al. 2000). VP4 also contains the tripeptide, IDA, at amino acids 538–540, which is a ligand-binding motif for integrin α4β1 (Coulson et al. 1997); however, the functionality of this site has not been demonstrated. Integrin αvβ3 has also been shown to be involved in the cell entry of several rotavirus strains at a post-attachment step (Graham et al. 2003). Besides, cell-surface heat shock cognate protein, hsc70, has also been implicated as a post-attachment receptor for both NA-sensitive and NA-resistant rotavirus strains (Guerrero et al. 2002). Studies with polarized epithelial cell lines show that the viral entry of SA-dependent strains is restricted to the apical membrane, whereas SA-independent strains enter either apically or basolaterally (Ciarlet et al. 2001). It has been suggested that lipid rafts might play a role in the cell entry of rotavirus (Isa et al. 2004), probably serving as platforms to allow an efficient interaction of cell receptors with the viral particle (Manes et al. 2003; Lopez and Arias 2004).

Recently it has been shown that HBGAs can act as receptors or co-receptors for certain genotypes of the VP4 protein (Hu et al. 2012; Huang et al. 2012; Liu et al. 2012; Liu et al. 2013; Ramani et al. 2013). These findings are relevant from the host genetics point of view because populations with a high prevalence of the Lewis-negative genotype are insensitive to rotaviruses carrying VP4 P[8] genotypes, thereby decreasing the circulation of P[8] genotypes and lowering the efficiency of rotavirus vaccines carrying the P[8] genotype.

Rotavirus infection in polarized, fully-differentiated Caco-2 cells is followed by a defect in brush-border hydrolase expression (Jourdan et al. 1998). Sucrase-isomaltase activity and apical expression are specifically reduced by rotavirus infection without any apparent cell destruction (Jourdan et al. 1998). In addition, viral infection induces an increase in intracellular calcium concentration, damages the microvillar cytoskeleton, and promotes structural and functional injuries at the tight junctions in cell-cell junctional complexes of Caco-2 cells monolayers without damaging their integrity (Brunet et al. 2000; Obert et al. 2000).

2.7. The NSP4 Enterotoxin

The rotavirus nonstructural glycoprotein, NSP4, functions as an intracellular receptor that mediates the acquisition of a transient membrane envelope as sub-viral particles bud into the endoplasmic reticulum. It has been demonstrated that NSP4 binds intracellular DLPs, interacting with VP6 (Estes and Kapikian 2007). Many structural motifs or protein regions have been implicated in the NSP4 biological function. Amino acids 17–20 from the C-terminus are necessary and sufficient for inner capsid particle binding (O'Brien et al. 2000) and the region involved in the retention of the NSP4 protein into the endoplasmic reticulum has been mapped to be between amino acids 85 and 123 at the cytoplasmic region of the protein (Mirazimi et al. 2003). Residues

at positions 48–91, a region which includes a potential cationic amphipathic helix, are involved in membrane destabilization (Tian et al. 1996; Browne et al. 2000). Purified NSP4 or a peptide corresponding to NSP4 residues 114–135 induce diarrhea in young mice after an increase in intracellular calcium levels, suggesting a role for NSP4 in rotavirus pathogenesis (Tian et al. 1994; Ball et al. 1996; Horie et al. 1999; Rodriguez-Diaz et al. 2003). In vitro studies have shown that following rotavirus replication in cells, a functional 7-KDa peptide of NSP4 (amino acids 112–175) is released from virus-infected cells into the medium by a non-classic, Golgi-independent cellular secretory pathway (Zhang et al. 2000). This endogenously produced peptide binds to apical membrane receptors to mobilize intracellular calcium through phospholipase C signaling. Seo et al. (2008) reported that recombinant NSP4 can bind to the metal ion-dependent adhesion site (MIDAS) present on integrins α1β1 and α2β1 and activates intracellular signalling pathways that regulate spreading between cells. Rotavirus and NSP4 alone induce age-dependent diarrhea and chloride permeability changes in mice lacking the cystic fibrosis transductance regulator (CFTR) (Morris et al. 1999). These results indicate that NSP4 is a novel secretory agonist, because the classical secretagogues carbachol and the diterpene forskolin that induce chloride changes by activating cyclic adenosine monophosphate, instead of by mobilizing $[Ca^{2+}]_i$ as a secondary mediator, fail to cause disease in CFTR knockout mice (Morris et al. 1999).

It has been suggested that NSP4 may directly inhibit the functioning of the cellular Na^+-dependent glucose transporter SGLT-1 (Halaihel et al. 2000). In addition, extracellular and/or intracellular NSP4 may contribute to diarrheal pathogenesis by altering the dynamics of intracellular actin distribution and intercellular contacts (Ciarlet and Estes 2001). NSP4 can affect the cytoskeleton in polarized epithelial cells, but how these pleiotropic properties of NSP4 influence its function in morphogenesis or pathogenesis remains unclear (Tafazoli et al. 2001; Zambrano et al. 2012).

It has been demonstrated that NSP4 stimulates the secretion of nitric oxide in cultured epithelial cells as well as *in vivo* (Rodriguez-Diaz et al. 2006; Borghan et al. 2007), indicating its role as an important mediator molecule in rotavirus infection. NSP4 also has adjuvant properties, suggesting a possible role in the innate immune response to rotavirus infection (Kavanagh et al. 2010). More recently it has been reported that NSP4 triggers the secretion of pro-inflammatory cytokines from macrophage-like THP-1 cells and nitric oxide from murine RAW 264.7 cells (Ge et al. 2013). Therefore, NSP4 acts as a pathogen-associated molecular pattern (PAMP) encoded by rotavirus and provides a mechanism for the production of pro-inflammatory cytokines associated with clinical symptoms of infection in humans and animals.

Another relevant finding on the pathophysiology of rotavirus and its enterotoxin has been the elucidation of the mechanism by which rotavirus, or NSP4 alone, activates the secretion of serotonin from enterochromaffin cells in the intestine. It was previously shown that serotonin played a relevant role

in rotavirus diarrhea (Lundgren et al. 2000) and with this new investigation the mechanism of serotonin secretion has been elucidated (Hagbom et al. 2011). Furthermore, a physiological explanation for rotavirus-induced vomiting has also been found (Hagbom et al. 2011).

The significance of immune response to NSP4 in protection against rotavirus infection in humans is still unknown, although it has been shown that NSP4 elicits both humoral and cell-mediated immune responses (Johansen et al. 1999; Ray et al. 2003; Rodriguez-Diaz et al. 2005; Vizzi et al. 2005). The carboxy end of NSP4 is immunodominant (Rodriguez-Diaz et al. 2004) and NSP4 is also able to elicit protective antibodies in the mouse model (Hou et al. 2008), reinforcing the idea that there is a relevant role of NSP4 in immunological protection from rotavirus infection in humans.

3. ASTROVIRUSES

Human astroviruses are non-enveloped viruses with a positive-sense, single stranded, polyadenylated RNA genome between 6.4 and 7.7 kb in length (Matsui 1997). Astroviruses are members of the *Astroviridae* family, divided into two genera: *Mamastrovirus* (MAstVs) and *Avastrovirus* (AAstVs) infecting mammalian and avian species, respectively. So far, astroviruses have been isolated from 31 species of mammals, 14 of them corresponding to different species of bats, which are thought to act as a reservoir (Chu et al. 2008; Zhu et al. 2009; Xiao et al. 2011). The recent discovery of many novel mammalian and avian astroviruses highlights the fact that astrovirus species cannot be established based solely on the host species. For recent update on taxonomic scheme of astroviruses, refer to the ICTV *Astroviridae* Study Group (Bosch et al. 2010).

The "classical" human astroviruses were classified into eight serotypes (HAstV-1 to HAstV-8) according to the antigenic reactivity of the capsid proteins (Lee and Kurtz 1982; Lee and Kurtz 1994; Taylor et al. 2001). Although the two recently identified groups MLB and VA/HMO astroviruses (Finkbeiner et al. 2009a; Finkbeiner et al. 2009b; Kapoor et al. 2009) infect humans, they are extremely genetically divergent from the classical human astroviruses (HAstV). These new strains are genetically closer to animal astroviruses of mink or sheep, than to HAstV, and thus must be considered different *Mamastrovirus* species infecting the same host (Bosch et al. 2010). Phylogenetic characterization of some of these viruses has shown that a single host species may be susceptible to infection by divergent astroviruses. This is the case of the recently described new human astroviruses (HAstV-MLB1 and MLB-2; HMOAstVs A, B, and C, and HAstV-VA1 and VA2) after metagenomic surveillance studies, which were found in patients suffering from gastroenteritis but are genetically unrelated to the classical eight serotypes of humans (HAstV-1 to HAstV-8) (Finkbeiner et al. 2008a;

Finkbeiner et al. 2008b; Finkbeiner et al. 2009a; Finkbeiner et al. 2009b; Kapoor et al. 2009). In fact, the number of astroviruses associated with humans has nearly doubled within the last few years. The term HAstV-VA/HMO has been proposed to refer to these new astroviruses (Bosch et al. 2014). So far, up to four VA/HMO strains have been described (Jiang et al. 2013). The discovery of novel astroviruses in human specimens reveals that there is greater diversity amongst astroviruses than was previously assumed considering the high level of similarity between human astroviruses 1 and 8 (Finkbeiner and Holtz 2013), raising many questions in regard to viral tropism, epidemiology, and potential link to disease.

Astroviruses produce infections mainly in young children, although illness rates increase again in the elderly (Lewis et al. 1989). However, they also cause disease in adults and immunocompromised patients (Cubitt et al. 1999; Coppo et al. 2000). Persistent gastroenteritis in children with no background disease has also been reported, mainly in association with serotype 3 strains (Caballero et al. 2003). Astrovirus infections occur worldwide and their incidence in children with gastroenteritis in both developing and developed countries ranges from 2 to 9 % (Gaggero et al. 1998; Bon et al. 1999; Mustafa et al. 2000; Guix et al. 2002).

Astroviruses are transmitted by the fecal-oral route, and outbreaks have been associated with consumption of sewage-polluted shellfish and ingestion of water from contaminated sources (Pinto et al. 1996; Pinto et al. 2001). They are frequently shed in stools in significant numbers at the onset of illness. In contrast to caliciviruses, astroviruses replicate in vitro in cell lines, which has allowed detailed studies of the viral replication cycle (Matsui 1997; Willcocks et al. 1999; Matsui et al. 2001). Human astroviruses were originally isolated in HEK cells and subsequently adapted to grow in LLCMK2 cells with trypsin-containing media, although without demonstrable cytopathic effects (CPE). The colonic carcinoma cell line, Caco-2, in contrast to LLCMK2 cells, are directly capable of infection with fecally derived astrovirus and display CPE as early as 2 days post-infection (Willcocks et al. 1990; Pinto et al. 1994).

3.1. Structure of the Virion

Astroviruses are generally described to have a diameter of 28 nm (Madeley 1979), although it may vary depending on the source of virus and the method of preparation for EM (Woode et al. 1984). More detailed ultrastructural analysis of human astrovirus serotype 2 grown in LLCMK2 cells in the presence of trypsin revealed particles with icosahedral symmetry and an array of spikes emanating from the surface (Risco et al. 1995). These particles had an external diameter on negatively stained preparations of 41 nm (including spikes). They did not display the star-like surface appearance characteristically found on fecally shed virus. The star-like morphology was inducible after alkaline treatment (pH 10), and further alkalinization (pH 10.5) led to particle disruption. Intact virions generally band at densities of 1.35–1.37 g/ml in CsCl (Caul and Appleton 1982), although banding at densities of 1.39–1.40 have also been

reported (Konno et al. 1982; Midthun et al. 1993). Astroviruses band at a density of 1.32 g/ml in potassium tartrate-glycerol gradient, which preserves the structural integrity of the virus better than CsCl (Ashley and Caul 1982).

3.2. Genome and Proteins

The RNA genomes of several cell culture-adapted human astrovirus strains have been cloned and sequenced (Jiang et al. 1993a; Lewis et al. 1994; Willcocks et al. 1994; Geigenmuller et al. 1997), providing new perspectives on their molecular biology. The organization of the genome includes three long ORFs, designated as ORF1a, ORF1b and ORF2. The length of the three ORFs varies among different viral strains and the largest variation in ORF1a is due to common insertions and deletions (in/del regions) in its 3′ end (Willcocks et al. 1994; Guix et al. 2005). ORF1a (~2700 nt) is located at the 5′ end of the genome and contains transmembrane helices, a 3C-like serine protease motif, a putative protease-dependent cleavage site, and a nuclear localization signal (Jiang et al. 1993a; Willcocks et al. 1994). ORF1b (~1550 nt) contains a RNA-dependent RNA polymerase motif, whereas ORF2 encodes the structural proteins (Matsui et al. 2001).

The nonstructural proteins of the virus are translated from the genomic viral RNA as two polyproteins (Jiang et al. 1993a). One of them contains only ORF1a and the other includes ORF1a/1b, and is translated via a –1 ribosomal frameshifting (RFS) event between ORF1a and ORF1b (Jiang et al. 1993a). Both proteins are proteolytically processed generating a variety of polypeptides, although it is unclear whether the viral protease is responsible for all the cleavages (Geigenmuller et al. 2002a; Geigenmuller et al. 2002b; Kiang and Matsui 2002). An additional ORF (ORF-X) of 91–122 codons, contained within ORF2 in a +1 reading frame, has been described in all HAstVs and some other mammalian viruses (Firth and Atkins 2010). Its initiation codon is located 41–50 nucleotides downstream the ORF2 AUG and might be translated through a leaky scanning mechanism. It remains to be determined whether the putative protein encoded in ORF-X is synthesized in HAstV-infected cells and what is its significance for virus replication (Méndez et al. 2013).

A subgenomic RNA of ~2.8 kb that contains ORF2 is found in abundance in the cytoplasm of astrovirus-infected cultured cells (Monroe et al. 1991; Monroe et al. 1993). The subgenomic RNA (approximately 2400 nucleotides) is translated as a 87 kDa capsid precursor protein that is believed to give rise to three to five smaller, mature capsid proteins in a process that involves trypsin and a putative cellular protease (Monroe et al. 1991; Bass and Qiu 2000; Mendez et al. 2002). The 87 kDa capsid protein is rapidly converted intracellularly to a 79 kDa form, which is found in smaller amounts in the cell supernatants. Bass and Qiu (2000) identified a trypsin cleavage site in a highly conserved region of the ORF2 product (Bass and Qiu 2000). Trypsin-free particles were minimally infectious in Caco-2 cells but became highly infectious after trypsin, but not chymotrypsin, treatment. This trypsin-enhanced infectiv-

ity correlated with conversion of the 79-kDa capsid protein into three smaller peptides of approximately 26, 29 and 34 kDa. However, the apparent molecular weight reported for the smaller mature proteins depends on the astrovirus serotype and whether the virus studied was derived from infected cultured cells or from stools (Matsui et al. 2001).

Astrovirus virus-like particles (VLPs) have been generated by cloning the cDNA corresponding to ORF2 from a human astrovirus serotype 2 virus into a vaccinia virus vector (Dalton et al. 2003). Protein composition of these purified VLPs revealed no substantial difference from that of authentic astrovirus virions when analysed by Western blotting. Trypsin cleavage seemed to be necessary to process the capsid polyprotein into the mature structural proteins. The three virological features which altogether distinguish astroviruses from other positive ssRNA viruses are: the location of the ORF1a and ORF1b encoding regions for non-structural proteins at the 5′ end of the genome and the presence of the ORF2 structural protein encoding region at the 3′ end; the use of a RFS mechanism to translate the RdRp encoded in ORF1b; and the lack of a helicase domain, characteristic of the positive ssRNA viruses with genomes larger than 6 kb. The presence of a VPg protein linked to the 5′-end of the astrovirus genome has also been speculated for many years but its existence has only recently been confirmed for human astroviruses (Fuentes et al. 2012).

4. ENTEROVIRUSES

4.1. Polioviruses

Enteroviruses replicate in the gastrointestinal tract, but the resulting infection is frequently asymptomatic. Symptoms, when they occur, range from paralysis to fever. Enteroviruses, named after the site of replication, rarely cause gastroenteritis. In many cases the enterovirus isolated might merely have been a passenger virus unrelated to the disease (Melnick 2001). The capsids of enteroviruses (family *Picornaviridae*) are composed of four structural proteins (VP1-VP4) arranged in 60 repeating protomeric units with icosahedral symmetry. Among the family members, the capsid proteins are arranged similarly, but the surface architecture varies. These differences account for not only the different serotypes but also the different modes of interaction with cell receptors. The basic building block of the picornavirus capsid is the protomer, which contains one copy of each structural protein. The capsid is formed by VP1 to VP3, and VP4 lies on its inner surface. VP1, VP2, and VP3 have no sequence homology, yet all three proteins have the same topology: they form an eight-stranded antiparallel "β-barrel" core structure (Racaniello 2007). The external loops that connect the beta strands are responsible for differences in the antigenic diversity among the enteroviruses. Neutralization sites are more densely clustered on VP1.

The structures of many human picornaviruses have been resolved and show that they share a number of conserved structural motifs. For example, the capsids of polio- and coxsackieviruses have a groove or canyon surrounding each fivefold axis of symmetry. In contrast, cardioviruses and aphthoviruses do not have canyons (Racaniello 2007). Immediately beneath the canyon floor of each protomer is a hydrophobic pocket occupied by a lipid moiety. These molecules, termed pocket factors, have been shown to stabilize the capsid and their removal from the pocket is a necessary prerequisite to uncoating. The pocket factors have been proposed, from the electron densities and uncoating studies, to be short chain fatty acids (Smyth et al. 2003).

The positive-sense RNA genome is approximately 7.4 kb long and serves as a template for both viral protein translation and viral replication. The 5′ end is covalently linked to a VPg protein. The genome is organized into a long (~740 nucleotide) nontranslated region (5′NTR), which contains the IRES and precedes a single ORF. The ORF is subdivided into three regions, P1 to P3. The P1 region codes for the structural proteins. The P2 and P3 regions encode the nonstructural proteins, essential for viral replication (2A-C and 3A-D). Translation of the ORF gives rise to a single large polyprotein that is posttranslationally modified by virus-encoded proteases. Immediately downstream of the ORF is the 3′ noncoding region (3′NTR; which plays a role in viral RNA replication) and a terminal poly(A) tail (Racaniello 2007).

Human poliovirus, including three serotypes (1–3), is considered a species within the genus *Enterovirus*. A redefinition of the criteria for species demarcation within the genus *Enterovirus* based on molecular and biochemical characteristics has recently been issued by the International Committee on Taxonomy of Viruses (ICTV). Based on these criteria, the genus *Enterovirus* now encompasses 12 species: enteroviruses A through J, and rhinoviruses A through C, according to the Ninth Report of the ICTV (Knowles et al. 2012a). Enteroviruses can cause in humans illnesses ranging from benign upper respiratory infections to severe meningitis and encephalitis. During the summer 2014, an unprecedented disease burden due to enterovirus-D68 (EV-D68) infections was reported from the USA (Midgley et al. 2014).

Poliovirus infections, once responsible for high morbidity and mortality worldwide, are now under control, and their eradication is a priority for the World Health Organization (WHO). Since the Global Polio Eradication Initiative was launched by WHO, the number of polio cases has fallen by over 99 %, from an estimated 350,000 cases in 1988–1919 in 2002. In the same time period, the number of polio-infected countries was reduced from 125 to 7. At present, polio is endemic in three countries: Afghanistan, Nigeria, and Pakistan. Until poliovirus transmission is interrupted in these strongholds, all countries remain at risk, as shown not only by a recent outbreak in Syria, but also in recurrent outbreaks across sub-Saharan Africa (Anonymous 2014). There is a historic opportunity to stop transmission of poliovirus. If the world seizes this opportunity and acts immediately, no child will ever again know the effects of this devastating disease (Dowdle et al. 2001).

4.2. Kobuviruses

Aichivirus, a cytopathic small round virus, was isolated for the first time in 1989 from fecal samples of patients involved in an oyster-associated gastroenteritis outbreak (Yamashita et al. 1991). Since then, several Aichiviruses have been isolated in BS-C-1 cells from patients with gastroenteritis (Yamashita et al. 1995). The virus is commonly found in outbreaks of gastroenteritis in Japan and is often associated with the consumption of oysters (Yamashita et al. 1995). Genetic analyses of Aichivirus revealed that it belongs to the *Picornaviridae* family, but that it is different from any other genus within this family (Yamashita et al. 1998). It was proposed by the ICTV that this virus be assigned to a new genus named *Kobuvirus* (King et al. 1999). "Kobu" means knob in Japanese, which was suggested due to the characteristic morphology of the virion. Currently, the existing species within the genus *Kobuvirus* have been renamed as follows: *Aichi virus* as *Aichivirus A*, *Bovine kobuvirus* as *Aichivirus B* and the porcine kobuvirus as *Aichivirus C* (Knowles et al. 2012b).

Aichivirus can be grown in BS-C-1 and Vero cells, producing CPE characterized by detachment of the cells. By negative staining and electron microscopy the virion shows a rough surface and measures around 30 nm in diameter. Three capsid proteins of 42, 30 and 22 kDa have been described (Yamashita et al. 1998). Aichi virus possesses a genome of single-stranded RNA of 8280 nucleotides, excluding a poly(A) tract. It contains a large ORF with 7299 nucleotides that encodes a polyprotein precursor of 2432 amino acids. The ORF is preceded by 744 nucleotides and followed by 237 nucleotides and a poly(A) tail. The precise secondary structure of the 5′ non-translated region (5′-NTR) has not been defined although an IRES similar to that of other members of the *Picornaviridae* family has been reported (Sasaki et al. 2001). The complete nucleotide sequence of Aichivirus (GenBank accession no. AB010145) has been determined (Yamashita et al. 1998).

Aichiviruses (AiVs) are subdivided into three genotypes (A, B, and C) based on differences in the nucleotide sequences at the 3C–3D junction (Yamashita et al. 2000; Ambert-Balay et al. 2008). Yamashita et al. (1998) showed that the VP0 capsid protein is expressed in the mature AiV particles and does not undergo cleavage (Yamashita et al. 1998), in contrast to other picornaviruses. Also the 2A and leader (L) proteins of Aichivirus have no protease or autocatalytic motifs as documented for other picornaviruses and their function remains unknown (Yamashita et al. 1998).

The organization of the deduced amino acid sequence of the polyprotein encoded by the Aichivirus genome is analogous to that of the other picornaviruses. Preceded by the L protein, there is a P1 region corresponding to the structural proteins (VP0, VP3 and VP1, with molecular weights of 42, 30 and 22 kDa, respectively), followed by the P2 and P3 regions which contain sequences encoding nonstructural proteins (2A-C, 3A-D). The 2A protein of Aichivirus contains conserved motifs that are characteristic of the H-rev107 family of cellular proteins involved in the control of cellular proliferation (Hughes and Stanway 2000). Amino acid sequences of the 2C, 3C and 3D

regions are well aligned with the corresponding sequences of other picornaviruses. The 3B protein corresponds to the VPg protein and the 3C protein is the protease which contains conserved motifs characteristic of all picornaviruses (Yamashita and Sakae 2003). The relationship of kobuviruses to other picornaviruses has been analysed based on the 3D amino acid sequence of the RNA polymerase within the polyprotein sequence (Hughes 2004).

Recently, a structurally distinct IRES was described (a fifth class of IRES) in members of *Kobuvirus,* as well as in *Salivirus* ("stool Aichi-like" virus) and *Paraturdivirus*, new genera of the *Picornaviridae* (Sweeney et al. 2012). Kobuviruses associated with infections in cattle and swine have been isolated and characterized (Yamashita et al. 2003; Reuter et al. 2009). By next generation sequencing, canine kobuviruses have also been detected in dogs in the USA (Kapoor et al. 2011; Li et al. 2011) and Europe (Carmona-Vicente et al. 2013). Evidence for a role of the virus in disease is lacking, although high seroprevalence in both dogs and cats suggests that infection is common (Carmona-Vicente et al. 2013). Other proposed kobuvirus species are mouse kobuvirus (MKoV) and sheep kobuvirus (SKV) (Reuter et al. 2011).

5. HEPATITIS A VIRUS

Hepatitis A virus (HAV), the prototype of the genus *Hepatovirus* in the family *Picornaviridae* (Minor 1991), is a hepatotropic virus which represents a significant problem for human health (Hollinger and Emerson 2001). The infection is mainly propagated via the fecal-oral route, and although transmission remains primarily from person-to-person, waterborne and foodborne outbreaks of the disease have been reported (De Serres et al. 1999; Hutin et al. 1999; Fiore 2004). The HAV was originally classified as enterovirus type 72 because its biophysical characteristics are similar to those of enteroviruses. However, later studies demonstrated that HAV nucleotide and amino acid sequences are different from those of other picornaviruses, as are the predicted sizes of several HAV proteins (Cohen et al. 1987b). This virus is difficult to cultivate in cell cultures and usually replicates very slowly without producing CPE, which has hampered its characterization. The virus is resistant to temperatures and drugs that inactivate other picornaviruses and is stable at pH 1. There is only a single serotype of human HAV with one immunodominant neutralization site (Lemon and Binn 1983), which is composed of closely clustered epitopes defined by two major groups of escape mutants that include residues 70, 71 and 74 of VP3 and residues 102, 171 and 176 of VP1 (Nainan et al. 1992; Ping and Lemon 1992). A second epitope is the glycophorin A binding site, represented by mutants around residue 221 of VP1 (Ping and Lemon 1992; Sanchez et al. 2004). Finally, there is a third and still undefined epitope, represented by escape mutants to the 4E7 monoclonal antibody (mAb) (Pinto et al. 2012).

A significant degree of nucleic acid variability has been observed among different isolates from different regions of the world (Robertson et al. 1992; Costa-Mattioli et al. 2001). The molecular basis of this genetic variability may be the high error rate of the viral RNA-dependent RNA polymerase and the absence of proofreading mechanisms (Sanchez et al. 2003). The HAV has a buoyant density of 1.32–1.34 g/cm^3 in CsCl and sediments with 156S during sucrose gradient centrifugation (Coulepis et al. 1982). Infectious viral particles with higher (1.44 g/cm^3) as well as lower (1.27 g/cm^3) buoyant densities have also been reported (Lemon et al. 1985).

Phylogenetic analysis based on a 168-base segment encompassing the VP1/2A junction region of the HAV genome has established the classification of human and simian isolates into six different genotypes (I-VI); genotypes I, II, III include human isolates (Robertson et al. 1992). About 80 % of the human isolates belong to genotype I, which has been subdivided into two subgenotypes, IA and IB (Fujiwara et al. 2003). This classification is based on a 168 nucleotide sequence at the VP1/2A junction (Robertson et al. 1992). Genotypes I, II and III contain subgenotypes defined by a nucleotide divergence of 7–7.5 %. Genetic diversity of HAV is evidenced by the emergence of new subgenotypes (Perez-Sautu et al. 2011).

5.1. The Genome

The genome is linear, single-stranded RNA of messenger sense polarity, approximately 7.5 kb in length, and a capsid containing multiple copies of three or four proteins named VP1, VP2, VP3 and a putative VP4 encoded in the P1 region of the genome (Racaniello 2007). The presence of this fourth protein, VP4, has been described repeatedly, but its apparent molecular weight (7–14 kDa) that has been reported does not correspond to those predicted from nucleic acid sequence data (1.5 or 2.3 kDa) (Weitz and Siegl 1998). The P2 and P3 regions encode nonstructural proteins associated with replication.

Detailed analysis of the HAV genome has been accomplished with cloned or RT-PCR–amplified cDNA. Cultured cells can be infected with RNA transcribed from cloned HAV cDNA (Cohen et al. 1987a). The genome differs from that of *Caliciviridae* in that the genes encoding the nonstructural and structural proteins are located at the 3′ end and 5′ end, respectively (Weitz and Siegl 1998). The HAV genome is divided into a 5′ nontranslated region (5′NTR) of 735 nucleotides, a long open reading frame of 6681 nucleotides encoding a polyprotein of 2227 amino acids, and a 3′ nontranslated region (3′NTR) 63 nucleotides in length.

The 5′NTR contains a cis-acting IRES, which initiates cap-independent translation directed to a particular AUG triplet several hundreds of nucleotides downstream (Glass et al. 1993; Brown et al. 1994; Ali et al. 2001; Borman et al. 2001; Kang and Funkhouser 2002). It has been demonstrated that the IRES is located between nucleotides 151 and 734, which is able to direct internal initiation of translation in a cap-independent manner (Brown et al. 1994).

However, a cap-dependent message effectively competes with the IRES of HAV (Glass et al. 1993). Translational efficiency of this IRES may be dependent on the availability of intact cellular proteins such as the p220 subunit of the eukaryotic initiation factor eIF-4 F, and requires the association between the cap-binding translation initiation factor (eIF4E) and eIF4G (Ali et al. 2001; Borman et al. 2001). The 5′-NTR is the most highly conserved region in the HAV genome among all strains sequenced to date, with a 95 % nucleotide identity. By contrast, the 3′-NTR region shows the highest (up to 20 %) degree of variability. The presence of a short cis-acting element has been described; it is 23 nucleotides long and specifically interacts with proteins involved in the establishment and maintenance of the persistent type of infection characteristic of most HAV isolates. Several unidentified cytoplasmic and ribosomal proteins in infected cells bind to the 3′ end of HAV RNA, indicating an intimate and dynamic interaction between host proteins and viral RNA (Kusov et al. 1996). The poly(A) tail is also involved in the formation of RNA/protein complexes.

Analogous to other picornaviruses, the coding region can be subdivided into P1, P2 and P3 subregions, which specify proteins 1A-D, 2A-C, and 3A-D, respectively. The polyprotein is further processed into the four structural and seven nonstructural proteins by proteinases encoded in and around the 3C region (Probst et al. 1998). Proteins 1A to 1D correspond to the structural proteins VP1-VP4. As in other picornaviruses, the 5′ end of HAV genome is covalently linked to VPg protein, which is specified by 3B, instead of the classical m7G cap structure (Weitz et al. 1986). The 2C gene carries a guanidine resistance marker and is assumed to play a role in viral RNA replication (Cohen et al. 1987b). The 3D region is the RNA-dependent RNA polymerase, which shows the highest degree of homology (29 %) with the corresponding sequence in the poliovirus type 1 protein (Cohen et al. 1987b). A region of 18 amino acids considered to be essential for an active polymerase is present in the 3D region. This sequence contains a conserved motif of two aspartate residues flanked by hydrophobic amino acids that might function as a GTP-binding domain (Kamer and Argos 1984). Replication efficiency seems to be controlled by amino acid substitutions in the 2B and 2C regions (Yokosuka 2000).

It has been proposed that HAV presents a higher codon usage bias compared with other members of the *Picornaviridae* family, which is characterized by the adaptation to use abundant and rare codons (Sanchez et al. 2003). A consequence of this special codon bias is an increase in the number of rare codons used. The critical role of HAV codon usage, and particularly of these clusters of rare codons of the capsid-coding region, has been shown in functional genomic studies during the process of adaptation of HAV to conditions of artificially induced cellular shut-off (replication in the presence of actinomycin D) (Aragones et al. 2010). It has also been suggested that this codon usage contributes to the low antigenic variability observed within the HAV capsid (Aragones et al. 2008). A total of 15 % of the surface capsid residues are

encoded by rare codons. These rare codons are highly conserved among the different HAV strains (Sanchez et al. 2003) and their substitution is negatively selected even under specific immune pressure (Aragones et al. 2008).

5.2. Proteins

The genomes of all picornaviruses encode a single polyprotein which is co- and post-translationally cleaved by virus-encoded proteinase(s). In contrast to well-characterized proteolytic events in the polyproteins of viruses within the genera *Enterovirus* and *Rhinovirus*, this processing has been difficult to characterize in the genus *Hepatovirus*. It has been shown that the primary cleavage of the HAV polyprotein occurs at the 2A/2B junction, which has been mapped by the N-terminal sequencing of 2B. This primary cleavage is mediated by the $3C^{pro}$ proteinase, the only proteinase known to be encoded by the virus (Martin et al. 1995; Gosert et al. 1996). The P1-2A capsid protein precursor is probably released from the nonstructural protein precursor (P3-2BC) as soon as $3C^{pro}$ is synthesized, as the full-length polyprotein has not been observed in these studies. A P1-2A precursor produced in a cell-free translation system has been shown to be cleaved in vitro by recombinant $3C^{pro}$ to generate VP0 (VP4-VP2), VP3 and VP1-2A (Malcolm et al. 1992). This VP1-2A polypeptide is unique to the hepatoviruses; it associates with VP0 and VP3 to form pentamers, intermediates in the morphogenesis of HAV particles (Borovec and Andersen 1993). The mature capsid protein VP1 is subsequently derived from the VP1-2A precursor later in the morphogenesis process. It has been hypothesized that the maturation of VP1 is dependent on $3C^{pro}$ processing of the VP1-2A precursor (Probst et al. 1998). However, it has also been shown, using recombinant vaccinia viruses expressing relevant HAV substrates, that $3C^{pro}$ is incapable of directing the cleavage of VP1 from the VP1-2A precursor. Therefore, maturation of VP1 could not depend on processing by $3C^{pro}$ proteinase (Martin et al. 1999).

5.3. Virus Replication

The main target cells for HAV replication are hepatocytes, although HAV antigen has also been detected in crypt cells of the intestine and Kupffer cells of the liver (Asher et al. 1995). Two cellular receptors for HAV have been proposed: the HAV cell receptor 1 (HAVCR1), which belongs to the T-cell immunoglobulin mucin family (Kaplan et al. 1996) and the asialoglycoprotein receptor, which binds and internalizes IgA-coated HAV complexes (Dotzauer et al. 2000). The general scheme of the replicative cycle of HAV is very similar to that of other picornaviruses (Racaniello 2007). After interaction with the receptor(s), the uncoating of the positive-sense RNA viral genome takes place. This process is extremely slow in HAV (at least in vitro with cell-adapted strains) taking several hours compared with the 30-min period common to most picornaviruses (Bishop and Anderson 2000). The HAV typically has a protracted and non-cytolytic replication cycle in cell culture and fails to shut down host cell metabolism (Lemon and Robertson

1993). Even after successful adaptation to growth in cell cultures, replication of HAV is a slow process that terminates in a state of persistent infection (de Chastonay and Siegl 1987). Cytopathic HAV strains have been recovered only from persistently infected cell cultures. Maximal levels of viral RNA synthesis can be detected at 24 h after infection and exponential production of progeny virus continues up to day 4 post-infection. Lysis of infected cells may become apparent within 3–9 days post-infection and yield of progeny virus rarely exceeds 10^7 $TCID_{50}$/ml (Siegl et al. 1984). Cap-independent translation of the viral genome takes place through the IRES within the 5′ noncoding region. The polyprotein is co- and post-translationally processed by the viral protease and the newly synthesized RNA-dependent RNA polymerase, as well as several membrane-interacting proteins, assemble at the 3′ end of the genomic RNA to start the synthesis of a negative-strand copy of the viral genome. Finally, after the structural proteins are assembled into capsid particles and the positive-strand RNA molecules are packaged, the newly synthesized virions are secreted across the apical membrane of the hepatocyte (Pinto et al. 2012).

6. HEPATITIS E VIRUS

The hepatitis E virus (HEV) is a nonenveloped, hepatotropic virus transmitted via the fecal-oral route or through uncooked animal products and contaminated water. It is classified as a *Hepevirus* in the family *Hepeviridae* (Emerson and Purcell 2007). Of the four known genotypes, genotype 3 is responsible for autochthonous infections in industrialized countries, with a seroprevalence in some European countries estimated as high as 22 %. Most of the HEV infections are caused by genotype 1 or 2 and occur in high-prevalence areas in East and South Asia where sanitary conditions are poor. Large outbreaks of HEV have also been reported from these areas, as from other areas of overcrowding and poor sanitation such as refugee camps (Ahmed et al. 2013). Chronic hepatitis E caused by HEV genotype 3 has been observed in immunosuppressed patients especially transplant recipients (Buffet et al. 1996). Serology is not sufficiently sensitive, especially in immunosuppressed patients, making PCR identification the preferred test for diagnosing active infection (Narayanan et al. 2005; Candido et al. 2012).

6.1. The Genome

The HEV contains a ~7.2-kb, single-stranded, positive sense, 5′-capped RNA genome. It consists of short 5′- and 3′ UTRs flanking three partially overlapping ORFs, namely, ORF1, ORF2, and ORF3 (Tam et al. 1991). The UTRs and a conserved 58-nucleotide region within ORF1 are likely to fold into conserved stem-loop and hairpin structures. These structures and a sequence closer to the 3′ end of ORF1, which has homology to the alphavirus junction

region, are proposed to be important for RNA replication (Purdy et al. 1993). The region between the end of ORF1 and start of ORF3/ORF2 appears to be complex and contains regulatory elements (Ahmad et al. 2011).

The cloning and sequencing of the HEV genome was first reported from cDNA libraries made from the bile of macaques experimentally infected with stool samples obtained from HEV patients (Reyes et al. 1990; Tam et al. 1991). Whole genomic sequences from various genotypes and different geographical isolates of HEV have also become available (Huang et al. 1992; Panda et al. 2000). Three RNA species of around 7.2, 3.7, and 2 kb, designated as the genomic and two subgenomic RNAs, respectively, were detected in the liver tissue of macaques experimentally infected with HEV (Tam et al. 1991). In this model, the 7.2 kb RNA was proposed to be translated into the ORF1 protein and the 3.7 and 2 kb subgenomic RNAs into ORF3 and ORF2 proteins, respectively (Ahmad et al. 2011). However, in stable Huh-7 cell lines made from functional HEV RNA replicons expressing the neomycin resistance gene from ORF2 and ORF3, only the genomic RNA and one subgenomic RNA were observed (Graff et al. 2006). This model was confirmed by intrahepatic inoculation of wild type and mutant genotype 3 swine HEV replicons into pigs (Huang et al. 2007). Mutation of AUG1 or the insertion of a T base, as in genotype 4, did not affect virus infectivity or rescue, but the mutation of AUG3 abolished virus infectivity.

Support for a single subgenomic RNA model also comes from PLC/PRF/5 cells that were either transfected with infectious genotype 3 viral RNA produced in vitro from a cloned cDNA or inoculated with fecal material containing genotype 4 HEV. The RNA isolated from these cells showed only the 2.2 kb subgenomic species, whose 5′ end mapped to nucleotide 5122 (Ichiyama et al. 2009). The viral negative-strand RNA is proposed to be a template for the synthesis of the positive-strand genomic and subgenomic RNAs, the latter from within the junction region in a primer-independent manner (Ahmad et al. 2011). The junction region of the negative-strand RNA is predicted to fold into a stable stem-loop structure and to play important roles in HEV replication (Cao et al. 2010).

6.2. Genetic Variants

By comparison of the full-genomes of HEV isolates, four major genotypes and several subtypes within each genotype have been identified (Lu et al. 2005). An avian HEV, initially considered as HEV genotype 5, is now proposed as a new species within the family *Hepeviridae* (Meng et al. 2011). A unique strain of HEV was identified from farmed rabbits in China, which shared 74–79 % nucleotide sequence identity to existing HEV strains and 46 % identity to avian HEV (Zhao et al. 2009). Recently, HEV-like viruses were also isolated and characterized from Norway rats (Johne et al. 2010), wild boars (Takahashi et al. 2011) and ferrets (Raj et al. 2012). The rabbit HEV appears to be a distant variant of genotype 3. Interestingly, a virus

isolated from cutthroat trout in California, but not associated with disease, bears only 18–27 % sequence similarity to avian or mammalian HEVs and was proposed as a new genus within the family *Hepeviridae* (Batts et al. 2011).

6.3. Proteins

The ORF1 nonstructural polyprotein of HEV is 1693 amino acids (~180 kDa) and contains several functional motifs and domains present in the nonstructural proteins of other positive-stranded RNA viruses. The ORF1 encodes a nonstructural polyprotein with four predicted functional domains, designated as methyltransferase (MeT), papain-like cysteine protease (PCP), helicase (Hel) and RNA-dependent RNA polymerase (RdRp). Besides these regions, two other regions designated X (and also called the macro domain) and Y, share significant homology with nonstructural proteins of other positive-strand RNA viruses. A polyproline region (PRP) or hypervariable region upstream of the macro domain may act as a flexible hinge. It is not entirely clear whether the ORF1 polyprotein is processed into biochemically distinct units, as is the case with other positive-strand RNA viruses. When expressed in mammalian cells using recombinant vaccinia viruses, ORF1 yielded processed products of 78 and 107 kDa (Ropp et al. 2000), whereas its expression in *E. coli* or HepG2 hepatoma cells showed no processing (Ansari et al. 2000).

Macro domain proteins hydrolyze ADP-ribose 1"-phosphate (ADPR-1"P), a product of cellular pre-tRNA splicing associated with poly(ADP-ribose) polymerase-1 (PARP-1), suggesting some role in cellular apoptosis (Egloff et al. 2006). It has also been suggested that the viral macro domains could have a role in the viral RNA replication and/or transcription. Since the HEV macro domains can bind poly(ADP-ribose) in the presence of poly(A), they could recruit poly(ADP-ribose)-modified cellular factors to the replication complex while bound to viral polyadenylated RNA (Neuvonen and Ahola 2009).

The ORF2 protein is the viral capsid protein; the crystal structure of a truncated recombinant ORF2 protein has been elucidated, but the size of the protein in mature virions remains unknown. The full-length protein is composed of 660 amino acid residues. The capsid protein contains three linear domains: S, M, and P (Guu et al. 2009; Yamashita et al. 2009; Xing et al. 2010). Variations in the ORF2 domains could influence cellular or humoral immune responses. The M domain contains T cell epitopes (Aggarwal et al. 2007). It is also a potential receptor binding site, as it contains a sequence that is strictly conserved among all genotypes (Guu et al. 2009). The P domain forms dimeric spikes on the surface of the capsid (Meng et al. 2001; Yamashita et al. 2009) and contains neutralization epitopes (Riddell et al. 2000).

When expressed in animal cells in culture, non-glycosylated and glycosylated ORF2 proteins of 74 and 88 kDa were observed (Zafrullah et al. 1999; Graff et al. 2008). A genotype 3 ORF2 protein expressed using recombinant vaccinia virus was also glycosylated and localized to the ER, Golgi, and surface of infected cells (Jimenez de Oya et al. 2012). A truncated protein of 56 kDa

(amino acids 112–607) can self-assemble in insect cells to form virus-like particles of 23–24 nm in diameter (Li et al. 1997). The ORF2 protein localizes to the ER, and some of the protein is retrotranslocated to the cytoplasm through an ER-associated degradation pathway (Surjit et al. 2007). It has also been demonstrated that ORF2 activates the phosphorylation of the eukaryotic initiation factor 2a (eIF2a), an increased expression of the ATF-4 transcription factor, and activation of the pro-apoptotic gene CHOP (John et al. 2011). An infectious cDNA clone of HEV has been constructed that propagates efficiently in cultured PLC/PRF/5 cells (Yamada et al. 2009). Using this model the intracellular expression and secretion of an 83 kDa ORF2 protein was shown. Though these authors did not directly test glycosylation of the ORF2 protein, a size significantly larger than the predicted size of ~72 kDa, suggests this possibility (Ahmad et al. 2011).

Another controversial issue is the existence of two types of virions: non-enveloped virions found in fecal samples, and "enveloped" virions found in serum samples (Takahashi et al. 2008; Yamada et al. 2009). It was reported that the enveloped virus is associated with the ORF3 protein and lipids, but the structure is largely unclear. Further studies are required to evaluate this subject (Ahmad et al. 2011). The ORF3 protein is a small, phosphorylated protein of 113 or 114 amino acids, whose function(s) has not been fully defined. It is dispensable for replication of HEV in vitro in cell lines (Emerson et al. 2006), but is required for infection in the macaque model of experimental infection (Graff et al. 2005). This suggests that the ORF3 protein functions as a viral accessory protein probably affecting the host response. The ability of the ORF3 protein to interact with multiple cellular proteins suggests its potential role in optimizing the cellular environment for viral infection and replication. ORF3 is a versatile, multifunctional protein that activates the extracellular regulated kinase (Erk), a member of the MAPK family, and this depends upon its ability to bind and inactivate an Erk-specific MAPK phosphatase (Kar-Roy et al. 2004).

6.4. Replication

The primary site of HEV replication is the liver, with hepatocytes being the most likely cell type. However, non-hepatic cell lines such as A549 lung carcinoma cells or Caco-2 colon carcinoma cells also support in vitro replication of HEV. No cellular receptor for HEV has been identified yet. It has been suggested that HEV enters liver cells through receptor-dependent clathrin-mediated endocytosis, although other entry pathways are also feasible (Kapur et al. 2012). Hsp90 and tubulin appear to be involved in capsid protein intracellular trafficking (Zheng et al. 2010). The virus uncoats to release the viral RNA that is translated in the cytoplasm into nonstructural proteins including the RNA-dependent RNA polymerase that replicates the positive sense genomic RNA into negative sense transcripts. The latter then act as template for the synthesis of 2.2 kb subgenomic RNA as well as full-length positive viral genomes (Graff et al. 2006).

The ORF2 protein packages the genomic positive sense RNA into progeny virions. Probably the ORF3 protein, together with lipids, coats the viral particle during the budding process (Takahashi et al. 2008). Although HEV is a non-enveloped virus, its association with lipids, the subcellular localization of the ORF3 protein to endosomes (Chandra et al. 2008), and a requirement for its PSAP motif in the viral egress (Nagashima et al. 2011), suggests that HEV follows the vacuolar protein sorting (VPS) pathway for its release from infected cells. The molecular virology of HEV will become better understood with the development of replicon- and infection-based in vitro cell culture models.

7. ENTERIC ADENOVIRUSES

Adenoviruses are non-enveloped, icosahedral viruses with a diameter of 70–90 nm. Five genera are currently recognized within the *Adenoviridae* family: two genera (*Mastadenovirus* and *Aviadenovirus*) that have probably co-evolved with mammals and birds, two genera with a broader range of hosts (*Atadenovirus* and *Siadenovirus*) and *Ichtadenoviruses* infecting fish (Harrach et al. 2011). The adenoviruses are species-specific and generally replicate only in cells derived from their native host. Human adenoviruses are associated with a variety of infectious diseases affecting the respiratory, urinary and the gastrointestinal tracts and the eyes (Horwitz 2001). To date, 51 serotypes of human adenoviruses have been recognized, which are classified into six subgroups (A to F) based on immunological properties, oncogenicity in rodents, DNA homologies, and morphological properties (Harrach et al. 2011). Adenoviruses have a buoyant density in cesium chloride of 1.33–1.34 g/cm^3. The capsid is composed of 252 capsomeres, of which 240 are hexons and 12 are pentons. Inside the capsid is a single molecule of linear, double-stranded DNA (Shenk 2001).

The enteric adenoviruses were originally identified from stool samples of infants with acute gastroenteritis (Flewett et al. 1973b) and have been consistently associated with gastroenteritis in children through epidemiological and clinical studies (Uhnoo et al. 1983; Uhnoo et al. 1984). The enteric adenoviruses are responsible for 5–20 % of cases of acute diarrhoea in children (Uhnoo et al. 1984; Kotloff et al. 1989; Uhnoo et al. 1990; Bon et al. 1999) and are found in clinical samples throughout the year with little seasonal variation (de Jong et al. 1983).

Enteric serotypes 40 and 41 have been designated as subgroup F adenoviruses. They share the adenovirus group antigen and are distinguished from each other and from other non-enteric serotypes by serology and DNA restriction patterns (Wadell 1984). The enteric serotypes are shed in large numbers from the gut of infected patients and were originally described as being non-cultivable or 'fastidious' adenoviruses because they could not be cultivated in

routine cell cultures that generally supported the propagation of other adenovirus types. However, it was found that these viruses could be propagated in Graham 293 cells, a cell line of human embryonic kidney (HEK) transformed with adenovirus 5 early (E) region 1 (Graham et al. 1977; Takiff et al. 1981), although at lower levels than other serotypes. This suggests that E1 functions are poorly expressed and therefore it was postulated that the inability of adenovirus types 40 and 41 to grow on cell lines normally supportive for other adenovirus types was due to the relative inability of the adenovirus 41 E1A gene to transactivate other adenovirus 41 genes (Takiff et al. 1984; Van Loon et al. 1985a). The Graham 293 cell retains the E1A and E1B regions of the adenovirus genome covalently linked to the host DNA. The mechanism of facilitation of the growth of the EAd40 in 293 cells seems to be a function of the E1B-55 kDa protein (Mautner et al. 1989). Grabow et al. (1992) reported another cell line (PLC/PRF/5 or primary liver carcinoma cells) also supported an efficient propagation of laboratory strains of adenovirus types 40 and 41. Efficient replication of adenovirus types 40 and 41 has also been achieved in other cell lines, like Hep-2 cells, Chang conjunctiva cells and Caco-2 cells (Perron-Henry et al. 1988; Pinto et al. 1994).

The adenovirus 40 genome has been sequenced (Davidson et al. 1993) (GenBank accession. no. L19443) and described in detail (Mautner et al. 1995). The main differences between adenovirus 40 and the other human adenovirus serotypes are the presence of two distinct fibre genes, a single VA gene involved in late translation, and a highly divergent E3 region. The adenovirus 41 growth restrictions in cell cultures seem to be less severe than those of serotype 40, as a number of cell lines support the propagation of serotype 41 and not adenovirus 40 (de Jong et al. 1983; Uhnoo et al. 1983; van Loon et al. 1985b). The adenovirus 41 blockade in replication occurs within the early phase of the infectious cycle (Tiemessen et al. 1996). The ability of adenovirus 40 E1A encoded products (proteins 249R and 221R) for *trans*-activation has also been investigated, (van Loon et al. 1987; Ishino et al. 1988); it has been found that the adenovirus 40 E1A promoter does indeed contain transcription factor binding sites sufficient for *trans*-activation by the adenovirus 5 E1A 289R protein. It is possible that adenovirus 40 has evolved to use components of the RNA processing machinery that are unique to enterocytes (Stevenson and Mautner 2003).

It has been suggested that interferon (IFN) sensitivity could at least in part be responsible for the fastidious growth of species F human adenoviruses. Experiments in conjunctival cells suggest that these viruses are defective in their ability to circumvent the antiviral actions induced by IFN (Tiemessen and Kidd 1993). Highly differentiated Caco2 cells were used as a model to determine whether HAdV-40 is sensitive to the effects of type I and type II IFNs. Infection counts showed that HAdV-40 infection rates were reduced significantly when the cells were pre-treated with IFN-α, compared with those either mock-treated or treated with IFN following infection. Species F adenoviruses may have adapted to tissues that are restricted in their ability to mount

an inflammatory cytokine response (Sherwood et al. 2012). Hence, the use of intestinal cell cultures may lead to a better understanding of adenovirus 40 replication and pathogenicity.

8. SUMMARY

Foodborne and waterborne viruses cause acute gastroenteritis (caliciviruses–noroviruses and sapoviruses, rotaviruses, astroviruses, and enteric adenoviruses), hepatitis (hepatitis A virus and hepatitis E virus), and other diseases. Other enteric viruses like kobuviruses, coronaviruses, toroviruses, and picobirnaviruses also may cause diarrhea, although the causative role for some of these viruses in humans is controversial. In addition, next-generation sequencing technologies have allowed the discovery of new enteric viruses (novel astroviruses, kobuviruses, saliviruses, etc.). Human caliciviruses have been recognized as the leading cause of acute gastroenteritis outbreaks and sporadic cases in children and adults worldwide. Enteric caliciviruses belonging to the *Norovirus* and *Sapovirus* genera still remain refractory to routine cell culture propagation. This limitation has hampered our ability to investigate their biology, pathogenesis, and host immunity, although molecular approaches have provided new insights into these areas. The morphology, composition, and structure of several enteric viruses have been elucidated. Cryo-electron microscopy and x-ray crystallography have been crucial for this purpose. Biochemical and structural studies of virus-like particles produced by recombinant baculoviruses are contributing to a better understanding of the structure-function relationships of the capsid proteins. Viral genome organization is being clarified for all of these viruses, as well as their replication and gene expression strategies. Most of the proteins encoded by the viral genomes have been characterized and their functions identified. Sequence analyses of viral genes have been applied in molecular epidemiology and taxonomy studies. However, many questions still remain to be answered.

Reverse genetics and replicon systems have provided an important tool for the study of replication and gene expression of different enteric viruses. The MNV constitutes an excellent model to analyze HuNoV replication cycle in the laboratory. Biochemical characterization of viral interactions with cells and analysis of the functional properties of the viral proteins are providing a better understanding of the pathogenesis of enteric viruses. Rotavirus NSP4 was the first viral enterotoxin to be characterized. Several cell membrane molecules have been identified recently as being receptors or attachment ligands for different enteric viruses (i.e., integrins and hsc70 for rotaviruses, HBGA carbohydrates for noroviruses). Studies on human susceptibility to norovirus infections have characterized some resistant, non-secretor (Se-) individuals in the population, which is a breakthrough in our knowledge of norovirus-host interactions. Similarly, molecular analyses of orally transmitted viruses causing hepatitis are clarifying the phylogenetic relationships between these viruses and other viral genera, as well as their pathophysiological mechanisms.

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CHAPTER 4

Epidemiology of Food-Borne Viruses

Aron J. Hall

1. INTRODUCTION

The prototype norovirus strain, Norwalk virus, was first described in 1972 following investigation of an outbreak of gastroenteritis at an elementary school in Norwalk, Ohio (Kapikian et al. 1972). Five years later, similar small round virus-like particles were identified in a series of outbreaks of food poisoning associated with consumption of cockles in the United Kingdom in which no bacterial etiology could be identified (Appleton and Pereira 1977). Despite these ground-breaking reports of what would later be classified as noroviruses causing gastroenteritis and specifically foodborne disease outbreaks dating back nearly 4 decades, recognition of the role of noroviruses in foodborne disease has been relatively recent. Insensitive early diagnostic methods, including electron microscopy and serology, and inability to culture noroviruses *in vitro* were largely to blame until the development of PCR-based diagnostic methods in the 1990s. The subsequent widespread implementation of these highly sensitive assays in both research and public health practice helped fuel a rapid expansion of knowledge in the field and specifically define the role of noroviruses in foodborne disease (Widdowson et al. 2005). Over the last decade, continued improvements in surveillance have helped establish noroviruses as the leading cause of foodborne disease in the United States and characterize their broader epidemiology.

Aside from noroviruses, other viruses can cause foodborne illness, notably hepatitis A virus (Fiore 2004). Sapovirus, rotavirus, and astrovirus have also occasionally been implicated in foodborne disease outbreaks, although data on the role of these viruses in causing foodborne disease are quite limited (Gastanaduy et al. 2013b; Hall et al. 2013b; Karlsson and Schultz-Cherry 2013; Scallan et al. 2011). Strategies to control hepatitis A now focus primarily on vaccination, which is recommended for routine use in the United States (Fiore et al. 2006). As noroviruses account for 95 % of reported foodborne viral outbreaks and 99 % of all foodborne viral illnesses in the United States (Gould et al. 2013; Scallan et al. 2011), this chapter will focus on noroviruses, specifically their disease burden and outbreak surveillance.

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2. DISEASE BURDEN

2.1. Challenges and Methods to Estimating Burden

With publication of the landmark study by Mead et al. in 1999, noroviruses were recognized as the leading cause of foodborne illness in the United States (Mead et al. 1999). However, as recognized by the authors, the norovirus disease burden estimates generated in this analysis were based on limited data primarily from non-US sources. Despite numerous advances over the ensuing decade, several challenges to estimating norovirus disease burden in the United States remain (Yen and Hall 2013). Many of these challenges stem from the lack of a rapid and sensitive clinical assay for diagnosis of norovirus infections. Given the relatively non-specific symptoms that manifest from norovirus infection, which can resemble several other agents of acute gastroenteritis, laboratory confirmation is necessary for definitive diagnosis. However, laboratory testing for norovirus is typically performed using molecular methods available primarily in public health laboratories and usually conducted only in the context of a public health investigation. Thus, the typical approach to case-based surveillance of reporting by healthcare providers or clinical laboratories, as with common foodborne bacteria such as *Salmonella* and *Escherichia coli*, is not possible for norovirus. Furthermore, only about 2 % of patients with acute gastroenteritis seek healthcare and have stool specimens collected for diagnostic testing (Hall et al. 2011a). Given this low rate of healthcare utilization, there would be tremendous underreporting even if norovirus were to become a nationally notifiable disease. Finally, while norovirus specific codes exist in administrative data sources, such as health insurance claims and hospital discharge databases, they typically are used only when there is laboratory confirmation and thus are insensitive and unreliable.

Despite these limitations, multiple methodologic approaches have been undertaken to estimate the burden of norovirus disease. The most scientifically robust method is active population-based surveillance, which involves systematic identification and testing of all suspect cases of norovirus disease within a known catchment area. Community-based studies using this approach have been performed in the Netherlands and in the United Kingdom (de Wit et al. 2001; Tam et al. 2012), yielding critical data that have been extrapolated to several other countries. In the United States, active population-based surveillance has been conducted for medically-attended norovirus disease in pediatric populations, revealing that norovirus has surpassed rotavirus as the leading cause of severe pediatric acute gastroenteritis since the introduction of rotavirus vaccines (Payne et al. 2013).

The intensive resources required for active surveillance have limited its scope and has led to the pursuit of less costly alternatives. For example, a passive surveillance strategy that involves norovirus testing of specimens that have already been submitted for routine clinical purposes can still provide valuable information, particularly if derived from a defined catchment population and selected systematically, as in a study of a managed care population

in Georgia (Hall et al. 2011a). Another alternative is to estimate the fraction of acute gastroenteritis that is attributable to norovirus and apply that to rates of non-specific acute gastroenteritis incidence. This may be done through either direct multiplication, as in the case of the US foodborne norovirus burden estimates (Mead et al. 1999; Scallan et al. 2011), or through regression modeling. This latter approach involves development of time-series regression models that indirectly estimate the proportion of cause-unspecified gastroenteritis that is likely caused by norovirus. Reassuringly, these various approaches have yielded generally consistent estimates of norovirus incidence, providing confidence in both validity of the methods and the accuracy of the estimates (Hall et al. 2013a).

2.2. Burden in the United States

Since publication of the first US estimate of norovirus disease burden (Mead et al. 1999), there have been seven studies that provide estimates of norovirus disease incidence in the United States. A detailed description of these studies was published in 2013 and the summary estimates are shown in Fig. 4.1 (Hall et al. 2013a). Each year in the United States, norovirus causes on average 19–21 million illnesses, 1.7–1.9 million outpatient visits, 400,000 emergency department visits, 56,000–71,000 hospitalizations, and 570–800 deaths. Norovirus disease occurs year round but incidence increases during winter months and can surge by up to 50 % during years in which new strains emerge. The highest rates of norovirus-associated deaths occur in older adults aged ≥65 years (Hall et al. 2012a), while the highest rates of norovirus-associated medical care visits occur in children aged <5 years (Gastanaduy et al. 2013a; Lopman et al. 2011).

Two studies have attempted to estimate the fraction of norovirus disease attributable to foodborne transmission for the purposes of estimating burden

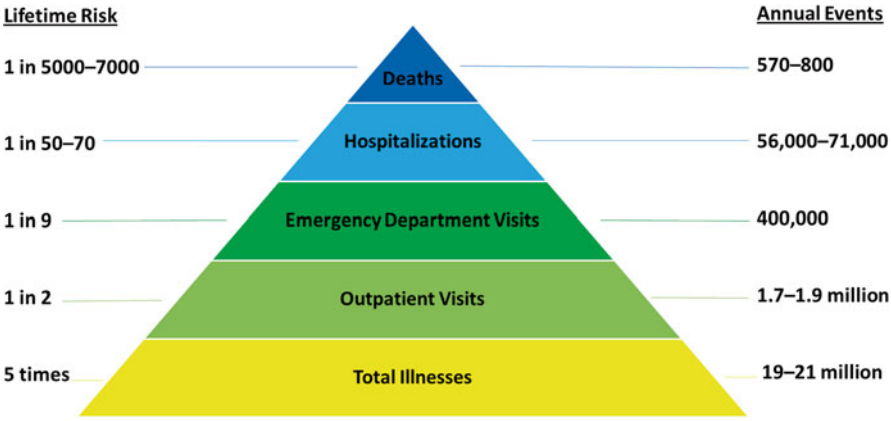

Figure 4.1. Annual burden and lifetime risk of norovirus illness and associated outcomes in the United States (adapted from Hall et al. 2013a).

in the United States. The first published by Mead et al. (1999) used an estimate of 40 % based on the proportion of norovirus outbreaks reported to CDC during 1996–1997 with a known transmission route, of which 47 % were foodborne (Fankhauser et al. 1998). The estimate was lowered to 40 % in recognition of the fact that foodborne outbreaks were more likely to be reported, although subsequent estimates suggest that this was still an overestimate of the fraction of norovirus that is foodborne. The updated estimate of foodborne disease in the United States by Scallan et al. (2011) used unpublished data from six states on norovirus outbreaks occurring during 2000–2005. Based on comparison of illnesses due to foodborne norovirus outbreaks relative to all norovirus outbreak-associated illnesses, the authors utilized a foodborne fraction for norovirus of 26 %. The use of this fraction led to norovirus ranking first in foodborne illnesses, second in foodborne hospitalizations, and fourth in foodborne deaths among all major pathogens assessed (Scallan et al. 2011).

2.3. Global Burden

Data on the global burden of norovirus disease have historically been limited to developed countries in which molecular methods of detection were available. However, the greatest burden of diarrheal disease occurs in developing countries particularly among young children, in which more than 25 % of deaths are attributable to acute gastroenteritis. A systematic review by Patel et al. (2008) of studies published between January 1990 and February 2008 identified 31 datasets from 22 countries that provided norovirus prevalence data meeting the inclusion criteria; only 11 of these dataset were from low-income settings. Through improved access to molecular diagnostics and efforts to leverage global surveillance networks for other diarrheal diseases, the number of studies assessing the role of norovirus in sporadic cases of acute gastroenteritis has increased dramatically in recent years.

Ahmed et al. (2014) recently updated the systematic review by Patel et al. (2008) and identified 150 additional articles with data on norovirus prevalence that were published during January 2008 through March 2014. Combining the results from these two reviews for meta-analysis, which included data from 48 countries, Ahmed et al. (2014) concluded that norovirus is associated with 18 % of acute gastroenteritis cases. The prevalence of norovirus was lower in high-mortality, developing countries (14 %) than in lower mortality settings (20 %), likely owing to the greater contributions of bacterial and parasitic agents associated with inadequate water, sanitation, and hygiene in developing countries. Additionally, norovirus detection rates tended to be higher among community (24 %) or outpatient settings (20 %) than in hospitalized patients (17 %). These findings suggest that norovirus is more often a cause of milder disease but the sheer frequency of infection still results in a significant burden of severe disease. The World Health Organization is leading efforts to combine the results of this meta-analysis with data on incidence and attribution of other foodborne disease agents to generate global estimates of the burden of foodborne disease (WHO 2015).

3. OUTBREAK SURVEILLANCE

3.1. Outbreak Detection Methods

Foodborne disease outbreaks are generally defined as the occurrence of a similar illness in two or more people associated with consumption of a common food (CDC 2015a). Detection of such events generally occurs through two primary methods. The first and most common method involves self-recognition among those affected following a common exposure, such as a restaurant meal or catered event. Health departments are then notified directly by the affected citizens or facility managers through complaint-based surveillance systems. A survey of local health departments reported that 93 % of them utilize complaint-based surveillance, which accounts for the detection of 69 % of all foodborne disease outbreaks (Li et al. 2011). In Minnesota, for example, virtually all foodborne norovirus outbreaks are detected through a well-established centralized complaint-based system; however, most of the complainants, including those subsequently confirmed to be infected with norovirus, are never linked to a recognized outbreak (Saupe et al. 2013).

Complaint-based detection of outbreaks is dependent upon the affected individuals having the opportunity and means to share their experiences after the fact so a specific event can be implicated. Social networks, therefore, play an important role in determining which outbreaks are recognized by this method. The increasing use of social media will likely aid in both the detection and subsequent investigation of foodborne disease outbreaks (Harris et al. 2014). Furthermore, the nature of the event itself can bias whether outbreaks detected through these social networks are then brought to the attention of public health. Public events involving retail or commercial food service may be more likely reported than private events in which one of the affected individuals is to blame. On the other hand, it can be difficult to link contaminated food served to unrelated individuals such as in restaurants unless there are multiple illnesses within a given dining party.

Several other factors specific to the affected individuals or the illness etiology can influence whether or not such events will actually be recognized. Larger events yielding a great number of people exposed, increase the odds that multiple cases will connect with one another and are therefore more likely to be detected than those involving just a few cases. Similarly, outbreaks are more likely to be detected when a high proportion of exposed people become ill (i.e., attack rate) due to high degree of contamination or to low infectious dose, for example. More dramatic or severe symptoms, such as acute projectile vomiting or bloody diarrhea might also increase the likelihood that affected individuals will recognize the illness as a possible foodborne disease. Of course, people may be reluctant to share such personal information beyond their immediate family when symptoms may be considered embarrassing or socially taboo to discuss.

The timing of illness onset relative to the implicated exposure (i.e., incubation period) also plays an important role in detection of an outbreak. People

tend to associate suspected foodborne illness with the last meal consumed; however, aside from chemicals and preformed bacterial toxins, most foodborne disease agents typically have incubation periods that are longer than a day (e.g., norovirus and *E. coli*) and some are on the order of weeks (e.g., hepatitis A virus, *Listeria*, and *Giardia*). Given issues with recall, longer incubation periods can result in decreased likelihood that a given individual will draw a connection between their illness and the actual exposure, let alone multiple linked persons doing so to detect an outbreak.

The second method by which foodborne disease outbreaks are detected involves subtyping of specific pathogens among individual cases of illness. In these situations, an ill individual seeks medical care, provides a clinical specimen (e.g., stool) from which a "notifiable" pathogen is identified. While state and local jurisdictions may vary in terms of reporting requirements, several foodborne diseases are designated as nationally notifiable in the U.S. by the Centers for Disease Control and Prevention (CDC), in collaboration with the Council for State and Territorial Epidemiologists (CSTE) (CDC 2015b). When such a notifiable pathogen is detected in a clinical specimen, it may then undergo further analysis to identify a genetic "fingerprint" of the pathogen. Networks of laboratories that perform these analyses can then upload the information into central databases and compare results. When clusters of multiple individuals infected with pathogens yielding the same genetic fingerprint are found, they can then be further investigated to determine if there is a common exposure and, therefore, an outbreak.

This pathogen-specific approach is the model used by PulseNet, the national molecular subtyping network for bacterial foodborne disease surveillance (Gerner-Smidt et al. 2013). Through subtyping methods such as pulsed-field gel electrophoresis (PFGE) and multiple locus variable-number tandem repeat analysis (MLVA), PulseNet detects multi-jurisdictional outbreaks of notifiable foodborne bacterial pathogens, such as *Salmonella* and Shiga toxin-producing *E. coli* (STEC). For norovirus, however, there are no routine clinical assays available for diagnosis of sporadic cases and such individual cases are not considered notifiable in most jurisdictions. Thus, this second method is generally not applicable for the detection of norovirus outbreaks, which are instead identified almost entirely through complaint-based systems.

3.2. Public Health Investigation

Once an outbreak is detected, the ensuing public health investigation typically involves a three-pronged approach comprised of epidemiologic, laboratory, and environmental components. The primary investigation goals are to characterize the extent of the outbreak and to identify the etiologic agent (if not already determined a priori), transmission route, and ultimately the source. In the United States, public health authority resides primarily with state and local governments; thus, state and local health departments lead most foodborne disease outbreak investigations. A typical outbreak

investigation often begins with an initial hypothesis generating stage, in which open-ended interviews are conducted with several affected individuals to gather information on the meals, events, or other potential exposures that may have resulted in the outbreak. If one or more hypotheses are developed through this process, the investigation may progress to an analytic study, such as a case–control or cohort study, to evaluate specific exposures among those that became ill and a comparison group. Stool specimens are collected from case-patients, ideally while they are still in the acute phase of illness, to maximize the likelihood of etiologic diagnosis. Depending on the suspected etiology and the diagnostic tests to be performed, different specimen types (e.g., bulk stool versus rectal swab) and transport media may be preferred (Lynch et al. 2006).

An environmental assessment of facilities in which the outbreak occurred is also critical to identify potential lapses in food safety practices, evaluate food workers for recent illness, and potentially collect food or environmental samples. Collection of environmental surface samples (i.e., swabs) are generally only recommended when there is specific epidemiologic evidence implicating a source and even then results are interpreted with caution given the limitations of these methods. Similarly, testing for noroviruses in foods during outbreak investigations is generally limited to shellfish, as there are no validated methods available for other food matrices. In situations where there are multijurisdictional issues, such as implication of a contaminated food product with wide geographic distribution, investigations are often performed in collaboration with CDC and other federal regulatory agencies (e.g., Food and Drug Administration [FDA] or U.S. Department of Agriculture Food Safety Inspection Service [USDA-FSIS]). These agencies can assist with trace-back and trace-forward, recalls of implicated products, and in the case of shellfish, food sample testing through the FDA Seafood Laboratory.

3.3. National Surveillance Systems

Since 2009, national surveillance for norovirus outbreaks occurs through two complementary epidemiologic and laboratory surveillance systems. The National Outbreak Reporting System (NORS) was launched by CDC in 2009 to integrate and enhance previously existent epidemiologic surveillance systems for foodborne and waterborne disease outbreaks (Hall et al. 2013c). Additionally, NORS provided the first national surveillance system for outbreaks resulting from direct person-to-person transmission, animal contact, contaminated environments, and other or unknown modes of transmission. Thus, NORS represents a comprehensive national surveillance system for all foodborne, waterborne, and enteric disease outbreaks occurring in the United States. Outbreaks are reported by state, local, and territorial health departments to the CDC through a web-based interface or direct data upload (CDC 2015c). Data captured through NORS include settings, transmission modes, case demographics, outcomes, and for foodborne disease outbreaks, information on the specific foods implicated and the factors contributing to their

contamination. NORS data are used to monitor trends in foodborne and other enteric disease outbreaks, including both temporal patterns and attribution of outbreaks to specific pathogens, settings, and contamination pathways (Hall et al. 2014).

Also launched in 2009, CaliciNet is the complementary laboratory-based surveillance system for norovirus outbreaks in the United States (Vega et al. 2011). Modelled on the success of PulseNet, CaliciNet functions similarly as a network of public health and regulatory laboratories performing sequence-based molecular genotyping of norovirus strains implicated in outbreaks (CDC 2015d). The sequence data along with basic outbreak characteristics are uploaded into a central database, which can then be used to track the molecular epidemiology of noroviruses, monitor for emergence of new strains, and potentially link outbreaks that may be associated with a common source (Vega et al. 2014). Participants in CaliciNet receive annual training and certification to ensure comparable diagnostic techniques are used throughout the network.

Building upon these national surveillance systems, CDC recently established a network of sentinel states to conduct enhanced norovirus outbreak surveillance. Beginning in August 2012, the Norovirus Sentinel Testing and Tracking (NoroSTAT) network provides near real-time assessment of norovirus activity and serves as an early warning system for the rest of the country (CDC 2015e). States participating in NoroSTAT are required to report suspected or confirmed norovirus outbreaks to CDC through both NORS and CaliciNet within seven business days of their notification. Those preliminary reports must include a limited set of required variables, including consistent outbreak identifiers in each system to enable linkage of the strain data collected through CaliciNet with epidemiologic data collected through NORS. The utility of this network was demonstrated immediately after implementation, by rapidly identifying and characterizing the emergence of the GII.4 Sydney strain during the 2012–2013 season (Leshem et al. 2013b).

3.4. Descriptive Epidemiology

Although norovirus outbreaks reported through national surveillance capture only about 0.2 % of the estimated 19–21 million annual cases of norovirus disease (Hall et al. 2014), they provide critical epidemiologic insights that can help guide prevention and control efforts. Notably, the advent of comprehensive surveillance for all pathways of norovirus outbreaks through NORS and CaliciNet has enabled improved estimation of the fraction of norovirus outbreaks that are attributable to foodborne transmission. Recent estimates from these systems indicate that 16–23 % of reported norovirus outbreaks are foodborne (Hall et al. 2014; Vega et al. 2014), which is consistent with estimates from similar outbreak surveillance systems in other developed countries (Verhoef et al. 2015) and is significantly lower than the 40 % figure used in the seminal foodborne disease burden estimates by Mead et al. (1999). As shown in Fig. 4.2, non-foodborne norovirus outbreaks occur overwhelmingly

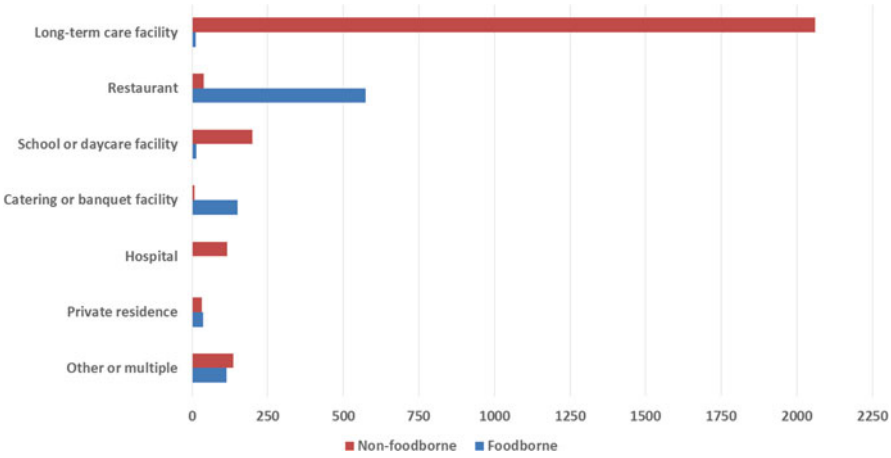

Figure 4.2. Number of norovirus outbreaks reported to the National Outbreak Reporting System by setting and transmission mode, United States, 2009–2012 (adapted from Hall et al. 2014).

in long-term care and other healthcare facilities (Hall et al. 2014), in which densely residing elderly populations are particularly vulnerable to infection and severe outcomes (Trivedi et al. 2012). In contrast, restaurants and other food service facilities are the most common setting for foodborne norovirus outbreaks, which most often affect younger adults (Hall et al. 2012b).

While most reported norovirus outbreaks are not associated with food, norovirus is still the leading cause of foodborne disease outbreaks in the United States, comprising approximately 50 % of all those reported with a single known cause (Gould et al. 2013; Hall et al. 2014). Food may become contaminated with norovirus at any point along the farm-to-fork continuum, but in >90 % of reported outbreaks, contamination occurs during final preparation and service (Hall et al. 2014). Of the outbreaks with data reported on the factors contributing to contamination, 50–70 % specifically implicated an infected food worker as the source, most of which involved bare-hand contact with ready-to-eat foods (Hall et al. 2012b; Hall et al. 2014). The specific food categories most frequently implicated in norovirus outbreaks are most often eaten raw, specifically leafy vegetables, fruits, and molluscan shellfish. In addition to contamination through handling by infected workers, these commodities can also become contaminated during production when grown with fecally-contaminated waters (Falkenhorst et al. 2005; Westrell et al. 2010; Le Guyader et al. 2012; Ethelberg et al. 2010).

Likely dating back to the initial description of "winter vomiting disease" (Zahorsky 1929), norovirus outbreaks exhibit pronounced winter seasonality, with approximately 80 % of all reported norovirus outbreaks occurring during November–April, and >50 % occurring in December–February specifically

Table 4.1 Predominant epidemiologic characteristics of norovirus outbreaks caused by genotype GII.4 strains versus non-GII.4 strains

Characteristic[a]	*GII.4 outbreaks*	*Non-GII.4 outbreaks*
Seasonality	Winter	Non-seasonal
Setting	Healthcare facilities	Non-healthcare (restaurants, schools)
Transmission	Person-to-person	Foodborne
Ages affected	≥65 years	≤65 years
Clinical severity	Elevated rates of hospitalization and death	Lower rates of severe outcomes

[a]Based on published descriptions of norovirus outbreaks (Desai et al. 2012; Leshem et al. 2013b; Matthews et al. 2012; Vega et al. 2014)

(Hall et al. 2014). This seasonal pattern is driven largely by outbreaks resulting from person-to-person transmission, whereas foodborne norovirus outbreaks occur more consistently throughout the year. These seasonal patterns likely result, at least in part, from human behaviors that result in more crowding and thus greater opportunities for direct transmission during colder months. However, there may also be virologic factors at play. While the GII.4 genotype predominates across all norovirus outbreaks, foodborne norovirus outbreaks are three times more likely to be caused by non-GII.4 genotypes (Vega et al. 2014). Thus, there may be genotype-specific characteristics that lead to differential propensities for transmission, such as environmental persistence and the natural history of the symptoms they produce. Based on general outbreak characteristics reported through national surveillance and published reports, distinct epidemiologic profiles emerge for GII.4 versus non-GII.4 norovirus outbreaks (Table 4.1).

Long-term temporal patterns of norovirus outbreaks are characterized by the periodic emergence of new viral strains, primarily those within the GII.4 genotype. Over the past 20 years, a new GII.4 strain has emerged every 2–4 years, likely driven by mutations to escape herd immunity, quickly replacing its predecessor in becoming the predominant strain causing outbreaks (Pringle et al. 2015). Sometimes, but not always, these emergent GII.4 strains can cause an increase in norovirus outbreaks, as was the case with the Farmington Hills strain in 2002–2003 and the Den Haag strain in 2006–2007 (CDC 2007; Zheng et al. 2010). However, emergence of the two most recent GII.4 strains, New Orleans in 2009 and Sydney in 2012, did not result in a recognized increase in the number of norovirus outbreaks (Leshem et al. 2013b; Yen et al. 2011). Strains of non-GII.4 genotypes, such as GII.12 and GI.6, have also periodically emerged in the United States with transient increases in their specific activities, although never reaching the overall epidemiologic significance of GII.4 strains (Leshem et al. 2013a; Vega and Vinje 2011).

4. SUMMARY AND CONCLUSIONS

Over the last decade, there has been a dramatic increase in the amount of data available on the epidemiology of noroviruses. Despite continued public perception of the predominance of bacteria (Cates et al. 2015), noroviruses are the leading cause of sporadic and epidemic foodborne disease in the United States. They exact a tremendous burden in terms of morbidity and use of public health resources in managing outbreaks. Although a variety of scenarios can result in norovirus contamination of foods, the most common involves bare-hand contact with ready-to-eat food by infected food service workers. Non-foodborne transmission likely results in even greater public health impacts and may be driven by both host factors and unique viral characteristics among norovirus strains. These improved estimates of incidence, outcomes, and attribution to specific sources provide the necessary grist to prioritize resources and can help guide targeting of interventions aimed at controlling the spread of noroviruses.

The foundation for norovirus prevention is built upon sound infection control and hygiene practices. Appropriate hand hygiene, including both washing and avoiding bare-hand contact with ready-to-eat food, along with environmental disinfection and exclusion of ill staff are critical to the prevention of both foodborne and direct person-to-person transmission of noroviruses (Hall et al. 2011b). However, implementation of these control measures has proven challenging and there are recognized limitations with compliance (Carpenter et al. 2013; Green et al. 2006). The high rate of infection in the community and the predominant role of infected food workers in foodborne disease transmission underscore the need to control endemic norovirus disease as a means of foodborne disease prevention. As such, norovirus vaccines hold the potential for significant public health impact and early clinical trials seem promising (Atmar et al. 2011; Bernstein et al. 2015). Further development of such vaccines and assessment of their potential impacts will rely on additional epidemiologic studies on the burden of disease and ongoing outbreak surveillance.

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CHAPTER 5

Epidemiology of Viral Foodborne Outbreaks: Role of Food Handlers, Irrigation Water, and Surfaces

Craig Hedberg

1. INTRODUCTION

Norovirus represents a considerable conundrum; although it is the leading cause of foodborne illness in the United States and other developed countries, most norovirus outbreaks are not attributable to food (Scallan et al. 2011). Of the foodborne outbreaks, most are associated with contamination of ready-to-eat foods by ill food handlers (Gould et al. 2013). The virus has a low infectious dose, is environmentally stable, and has a high degree of resistance to commercial sanitizers and disinfectants. Molecular characterization and sequencing of viruses associated with outbreaks has done much to deepen our understanding of the underlying epidemiology of norovirus and to link multiple case clusters to common contaminated food items, but case-based surveillance is limited by the lack of routine diagnostic testing in clinical laboratories (Vega et al. 2014; Verhoef et al. 2011).

The development of multi-agent, culture-independent diagnostic tests may usher in a new era of case-based norovirus surveillance. Identification of case clusters based on sequencing of viruses would greatly extend the range of outbreaks that can be detected (Mijovski et al. 2008). Improved surveillance and outbreak investigation methods could permit meaningful attributions of illnesses to transmission pathways and inform risk assessment models to guide development of risk mitigation strategies (Vega et al. 2014; Verhoef et al. 2011). The application of new technologies and the integration of surveillance activities at national and international levels may lead to better outbreak control and public health prevention measures. However, our understanding of the underlying epidemiology of norovirus outbreaks has not fundamentally changed in over 30 years of surveillance and outbreak investigations. The types of outbreaks currently making headlines reflect the same patterns that were recognized early on.

The primary routes of transmission for noroviruses are in many respects determined by the setting (Table 5.1). Increased understanding of norovirus strain characteristics demonstrates that there is variability in agent-host interactions that affect the frequency and magnitude of transmission across these settings (Verhoef et al. 2010; Petrignani et al. 2015). Much work remains to be

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Table 5.1 Primary transmission routes for noroviruses by setting and by characteristics of the settings

Setting	*Primary transmission route*	*Characteristics of setting that favor transmission route*
Facility of community with untreated water system	Waterborne	Fecal contamination of well or water system
Restaurants	Foodborne	Transient customer base with limited opportunities for environmental contamination and repeated exposures. Resident food handlers provide extended source of contamination during outbreaks
Institutional	Person-to-person, environmental	Resident population with many opportunities for environmental contamination and repeated exposures

done. For example, in the arena of foodborne transmission, important questions on the role of environmental contamination of irrigation water and food handling surfaces remain to be answered.

Noroviruses are the most common cause of viral foodborne outbreaks by a wide margin (Scallan et al. 2011). In the absence of specific diagnostic testing, they also serve as useful models for transmission of other viral agents. The only other foodborne virus that has considerable public health importance is hepatitis A virus (HAV), due to the potential severity of illness it causes and to the potential of interrupting transmission by the pre-exposure use of HAV vaccines and post-exposure use of immunoglobulins (Fiore 2004; Petrignani et al. 2014). Much of the present discussion of the role of food handlers, irrigation water and contaminated surfaces in the occurrence of viral foodborne outbreaks will focus on noroviruses. Where important distinctions need to be made for the epidemiology of HAV, these will be noted.

2. OUTBREAK DETECTION, INVESTIGATION, AND SURVEILLANCE

Foodborne outbreaks of norovirus are detected by the recognition of vomiting or diarrhea in a group of people within a few days of a common exposure at an event or establishment. This was true in the 1970s, when Norwalk virus was first identified as the cause of "winter vomiting disease", and it remains true 40 years later. For example, 202 confirmed or suspected foodborne norovirus outbreaks were reported by the Minnesota Department of Health (MDH) from 2000 to 2006 (Li et al. 2010). Of these, 189 were reported to

MDH from consumers complaining about illness associated with an establishment (53 %) or an event (40 %). Specimens collected during the investigation of these outbreaks and tested by MDH confirmed norovirus in 81 % of these reported outbreaks. However, because there is no routine clinical laboratory testing available, only 3 (1.5 %) foodborne norovirus outbreaks were reported by health care providers (Li et al. 2010).

The recognition of illness among defined groups leads to the frequent reporting of norovirus outbreaks in institutional settings such as long-term care facilities and schools. Of 4318 confirmed and suspected norovirus outbreaks reported to CDC's national outbreak reporting system (NORS) from 2009 to 2012, 2072 (48 %) were from long-term care facilities and 161 (4 %) were from schools (Hall et al. 2014). In contrast, 612 (14 %) outbreaks were reported from restaurants and 159 (4 %) from catering or banquet facilities. While foodborne outbreaks were reported in a wide range of settings, only 8 % of norovirus outbreaks in schools and less than 1 % in long-term care facilities were attributed to foodborne transmission. The vast majority of these outbreaks are due to person-to-person spread of the virus. In institutional settings, extended person-to-person or environmental transmission may occur over several weeks. Because of the apparent person-to-person transmission, the role of foodborne transmission may not be formally assessed. Only in restaurants and catering and banquet facilities, where 95 % of outbreaks were foodborne, was foodborne transmission the predominant mode of transmission reported (Hall et al. 2014).

While the vast majority of foodborne norovirus outbreaks are detected by consumer complaints, <10 % of complaints are associated with outbreaks (Li et al. 2010). In addition, relatively few complainants seek health care for their illness or get a specific diagnosis. Only 13 % of Minnesota callers, who complained about an outbreak from 2000 to 2006, visited a health care provider (Li et al. 2010). To address the lack of diagnostic information among complainants, the Minnesota Department of Health began testing callers who reported diarrhea or vomiting. Of 241 persons tested from October 2011 to January 2013, 127 (53 %) were positive for norovirus (Saupe et al. 2013). Thus, sporadic cases of norovirus appear to be the leading cause of illness among persons reporting suspected foodborne illnesses.

Suspected norovirus outbreaks are recognized by the characteristic presentation of sudden onset of vomiting and diarrhea among members of a group, 24–48 h following a common exposure (Turcios et al. 2006). Before the widespread availability of RT-PCR testing for noroviruses in public health laboratories, the etiology of norovirus outbreaks was rarely confirmed. For example, between 1993 and 1997, only 6 % of foodborne outbreaks with a confirmed etiology were attributed to viruses, and 68 % of all outbreaks were classified as having an unknown etiology (Olsen et al. 2000). A high proportion of these outbreaks had clinical and epidemiologic characteristics of norovirus (Hedberg et al. 2008). These characteristics included a median incubation period of 24–48 h, median duration of illness from 12 to 60 h, and vomiting in more than

50 % of cases. In outbreaks involving mostly adults where fewer than 50 % of cases reporting vomiting, vomiting was more commonly reported than fever (Turcios et al. 2006). These clinical characteristics were used by local public health officials to guide foodborne outbreak investigations in restaurants and were subsequently incorporated into surveillance case definitions (Hedberg et al. 2008). From 2001 to 2008, 46 % of confirmed foodborne outbreaks in the US were attributed to norovirus (Hall et al. 2012) of which 58 % were confirmed by laboratory testing in public health laboratories. From 2009 to 2012, 69 % of reported norovirus outbreaks were confirmed by laboratory testing (Hall et al. 2014).

The widespread availability of laboratory testing capacity in public health laboratories has had two important impacts. First, the ability to confirm the etiology of an outbreak encourages public health officials to more thoroughly investigate outbreaks and report their findings. For example, in foodborne outbreaks in restaurants investigated from 1982 to 1997, contributing factors reported from outbreaks with a known etiology were consistent with the known biology of the agent (Hedberg et al. 2008). Thus, improper holding times and temperatures were identified in 84 % of *Clostridium perfringens* outbreaks and poor personal hygiene was identified in 75 % of norovirus outbreaks. For outbreaks that were not confirmed by laboratory testing, improper holding was identified in 53 % of outbreaks with a diarrhea-toxin profile and poor personal hygiene was identified in 35 % of norovirus-like outbreaks (Hedberg et al. 2008). In comparison, for outbreaks that did not match a clinical profile, improper holding and poor personal hygiene were identified in only 30 and 20 % of outbreaks, respectively. Among all foodborne outbreaks reported to CDC from 1998 to 2008, contributing factors were reported for 58 % of 7998 outbreaks with a known etiology, but only 32 % of outbreaks with an unknown etiology (Gould et al. 2013). The percentage of outbreaks with a known etiology increased from 40 % in 1998 to 67 % after 2001. During this same time, the proportion of foodborne outbreaks attributed to norovirus increased from 28 to 50 % (Gould et al. 2013). Thus, public health surveillance data on contributing factors for foodborne norovirus outbreaks are considerably more robust today than they were 20 years ago.

The second major impact has been the increased understanding of the epidemiology of norovirus from genotyping and sequencing of viruses associated with outbreaks. Genotype GII.4 has been shown to be the most common cause of norovirus outbreaks worldwide, with the periodic emergence of new strains that replace circulating strains in a manner similar to the epidemiology of influenza viruses. GI viruses have been associated with waterborne and foodborne transmission (Lysén et al. 2009; Kroneman et al. 2013) while GII.4 viruses have been associated with outbreaks in long-term care facilities (Vega et al. 2014; Hall et al. 2013).

In the US, a laboratory-based surveillance network for norovirus outbreaks (Calicinet) was established in 2009 to provide national aggregation of molecular subtyping information on noroviruses along with epidemiologic

Table 5.2 Relative and absolute risk of foodborne transmission by genotype, US, 2009–2013[a]

Genotype	*No. foodborne outbreaks*	*No. reported outbreaks*	*Relative risk*	*Absolute risk*
GI	84	387	2.6	Reference
GII (excluding GII.4)	126	618	2.4	1.5
GII.4	239	2841	Reference	2.8
Total	449	3846	Relative and absolute risks are calculated in comparison to reference category	

[a]Adapted from Vega et al. (2014)

information and results of outbreak investigations. Of the outbreaks with a known route of transmission reported to Calicinet from 2009 to 2013, person-to-person transmission was reported in 84 % of outbreaks, and foodborne transmission was reported for 16 % (Vega et al. 2014). In an analysis of routes of transmission by genotype, several GI and GII genotypes were associated with an increased rate of foodborne transmission, while GII.4 was associated with a lower rate of foodborne transmission. The lower rate of foodborne transmission associated with GII.4 was due to its frequent occurrence in long term care facilities, and the preponderance of person-to-person transmission in these outbreaks. However, GII.4 norovirus strains accounted for 53 % of foodborne outbreaks. Thus, while on a relative basis GII.4 is less likely to be associated with foodborne disease transmission than other genotypes, on an absolute basis it is the most frequent cause of foodborne illness (Table 5.2) (Vega et al. 2014). Rather than being in any way deficient in its ability to cause foodborne illness, GII.4 strains appear to have enhanced capacity to spread from person-to-person.

The big gain to be made from sequencing noroviruses associated with outbreaks will be in the ability to link multiple apparently independent outbreaks to a common source (Verhoef et al. 2011; Maunula et al. 2009). Multi-state and international outbreaks make up a small fraction of all foodborne norovirus outbreaks. However, as has been demonstrated in Europe, with repeated detection of international outbreaks linked to frozen berries, real-time exchange of epidemiologic and molecular data could lead to the linkage of clustered outbreaks, in much the same way that PulseNet can currently link clusters of bacterial foodborne pathogens (Falkenhorst et al. 2005; Müller et al. 2014; Sarvikivi et al. 2012). In addition, the phylogeographic analysis of norovirus outbreaks could facilitate identification of contaminated food sources that are currently unrecognized (Verhoef et al. 2011).

Surveillance of HAV infections differs considerably from that of norovirus. Hepatitis A infections are typically diagnosed by serologic tests conducted in symptomatic cases. In addition, HAV can be detected by RT-PCR and

sequenced (Fiore 2004). Clusters of cases may be diagnosed by association with households, institutions, common events, or establishments. However, the incubation period of HAV may encompass several weeks and outbreaks are rarely identified through interviews of unrelated sporadic cases (Petrignani et al. 2014). Restaurants are frequently identified as potential sources of exposure following the diagnosis of HAV infection in a food worker. Although food handler infections are not rare, most do not result in transmission to patrons. Food handlers, who have poor personal hygiene, prepare ready to eat foods such as salads, and work during the early stages of their illness may contaminate foods that lead to transmission of virus to patrons (Fiore 2004).

3. ROLE OF FOOD HANDLERS

Humans are the only source for strains of norovirus associated with foodborne outbreaks. Thus, a human source of contamination is theoretically identifiable for all foodborne outbreaks, whether the contamination event occurs during primary production, processing, or at the point of service of food. However, the potential impact of the outbreak and the potential for prevention and control of transmission vary considerably across these settings.

From 2001 to 2008, an average of 365 foodborne outbreaks of norovirus per year were reported to CDC. Contamination at the point of service was identified for 85 % of outbreaks in which a single, simple food item was implicated (Hall et al. 2012). From 2009 to 2012 the number of foodborne norovirus outbreaks reported to CDC declined to an average of 252 per year (Hall et al. 2014). However, contamination at the point of service accounted for 92 % of outbreaks in which one specific food item was implicated. Differences in compilation of data and approach to analysis precludes a direct comparison between these studies, but throughout these time periods, infected food handlers accounted for most of the contamination at the point of service. Infected food handlers were identified as the source of contamination for 53 % of outbreaks from 2001 to 2008 and 70 % of outbreaks from 2009 to 2012 (Hall et al. 2014).

In the United States, an estimated 5.5 million foodborne norovirus illnesses occur each year (Scallan et al. 2011). An estimate of the burden of these illnesses that may be due to restaurant employees working while ill can be made from the expected occurrence of illness among restaurant employees and knowledge of the likelihood of their working while ill (Table 5.3). Based on published estimates for these parameters, as many as 2.2 million foodborne norovirus illnesses may be due to restaurant employees working while ill; this accounts for 75 % of restaurant-associated norovirus illnesses in the US (National Restaurant Association 2015; Scallan et al. 2011; Hall et al. 2012; Sumner et al. 2011).

Table 5.3 Estimated burden of norovirus in the United States due to infected restaurant employees working while ill[a]

Variable	*Estimate*
No. of employees working in restaurants	14 million
Rate of diarrheal illnesses, per person/year	0.6
Illnesses among restaurant employees	8.4 million
Percentage of diarrheal illness due to norovirus	11 %
Norovirus illnesses among restaurant employees	924,000
Percentage of food workers who work while ill	20 %
Norovirus infected restaurant workers who work while ill	185,000
Average no. ill per outbreak in a restaurant	12
Illnesses due to ill employees	2.2 million
Percentage of foodborne norovirus outbreak-associated illnesses due to restaurants	54 %
Percentage of restaurant-associated norovirus due to infected employees working while ill	75 %

[a]Estimates derived from National Restaurant Association (2015), Scallan et al. (2011), Sumner et al. (2011) and Hall et al. (2012)

Although infected food handlers account for most of the foodborne outbreaks in food service establishments, there is a growing awareness of transmission from food items prepared and served by asymptomatic employees (Franck et al. 2015). Food handlers returning to work shortly after recovering from clinical symptoms have long been recognized as being infectious (White et al. 1986). This has led to public health recommendations that food handlers stay home for at least 48 h after symptoms stop (Parashar et al. 1998). In Denmark, 11 (6 %) of 191 foodborne outbreaks analyzed from 2005 to 2011 were attributed to post-symptomatic food handlers who would have been subjected to the above-referenced exclusion recommendations (Franck et al. 2015). While experimental data demonstrate that infected persons can shed norovirus for more than a week after symptoms stop, there is little evidence that this extended period of post-recovery shedding has contributed to the recurrence of outbreaks in restaurant settings after control measures have been implemented (CDC 2011).

A more common context for foodborne transmission by asymptomatic food handlers has been the setting in which food handlers denied being personally ill but noted the occurrence of illness among household members. This accounted for 26 % of food handler-associated outbreaks in Minnesota from 1981 to 1998, and 33 % of food handler-associated outbreaks in Denmark from 2005 to 2011 (Deneen et al. 2000; Franck et al. 2015). An interesting twist on this theme led to the occurrence of a point source outbreak linked to a hotel kitchen in Ireland (Nicolay et al. 2011). Three norovirus positive but asymptomatic

food handlers prepared sandwiches that were associated with illness among guests of a luncheon. The only recognized contact these food handlers had with an ill person prior to the event was through the common use of a bathroom in which a vomiting event had occurred a week earlier (Nicolay et al. 2011).

Because transmission of norovirus among food handlers is frequently seen during outbreaks in restaurants, stool samples collected from food handlers during outbreak investigations are likely to identify asymptomatic infections (CDC 2007; Ozawa et al. 2007). It is difficult to systematically evaluate the role that these asymptomatic food handlers may play in initiating or propagating the outbreak. Similarly, in the absence of illness among food handlers or household contacts it may be difficult to confirm whether an asymptomatic food handler was the source of the outbreak, or may have been infected as a result of exposure to a food item that was contaminated when it arrived at the establishment. In approximately 5 % of outbreaks, illnesses among food handlers appear at about the same time as among guests (Hedberg et al. 2008; Franck, et al. 2015). In restaurant-associated outbreaks caused by *Salmonella* and *Shigella*, food handler infections have been linked to exposure to food items, including fresh produce items contaminated before the point of service (Medus et al. 2006; Naimi et al. 2003). This has been confirmed by identifying the same outbreak-associated strains in multiple establishments.

Until recently, the lack of laboratory-testing resources has limited the ability of public health agencies to make the linkage between establishments with norovirus outbreaks (Anderson et al. 2001). As genotyping and sequencing of noroviruses associated with outbreaks becomes a routine practice, widespread outbreaks caused by primary contamination of fresh produce items would be more readily detectable (Mathijs et al. 2012). Public health agencies responsible for outbreak investigations need to be aware of the characteristics that suggest that a contaminated food item is a likely source of an outbreak, and then take appropriate actions to identify the source of the outbreak (Table 5.4). Integrating outbreak investigation activities across multiple agencies is needed to identify multistate or international outbreaks from a common source (Verhoef et al. 2011). Environmental assessments conducted as part of these efforts are needed to elucidate the role of irrigation water in the epidemiology of norovirus.

4. ROLE OF IRRIGATION WATER

Water is an efficient vehicle for norovirus transmission. Although not as common as foodborne outbreaks, waterborne transmission of norovirus occurs as a result of contamination of both drinking water systems and recreational waters (Gallay et al. 2006; Gelting et al. 2005). Most outbreaks of foodborne norovirus attributed to contamination at the point of production involve shellfish that were harvested from waters contaminated by human

Table 5.4 Outbreak characteristics that suggest that a contaminated food item or ingredient is the likely source of an outbreak and should be subject to a traceback to identify the likely point of contamination

Outbreak characteristic	*Rationale for further investigation and traceback of source*	*Follow-up action*
Food handlers not ill or ill at same time as guests	Although food handlers with asymptomatic infections may be capable of contaminating food, in most outbreaks they have recently recovered from an illness, or live in a household with ill family members. If food handler illnesses occur at the same time as illnesses among guests it may suggest a common exposure to a contaminated food item served at the establishment	Traceback implicated or suspected food items, look for possible related outbreaks linked to same source
Implicated food item is fresh or frozen produce item that is ready-to-eat and served with minimal handling	Multi-state and international outbreaks have been associated with contaminated frozen berries. If the food item is minimally handled at the point of service it increases the likelihood that there was an earlier point of contamination	Traceback implicated or suspected food items, look for possible related outbreaks linked to same source
Outbreak cluster recognized	When multiple outbreaks are identified over a short time period in establishments that may share a common distributor or feature similar food items on their menus, it may indicate distribution of a common food item. This likelihood may be increased if clusters occur outside of the peak season for norovirus transmission	Link outbreak investigations to identify a potential common source
Uncommon genotype or novel sequence profile identified	If norovirus strains that are linked to outbreaks appear different from the prevailing strains circulating in the community (including strains associated with institutions or person-to-person transmission) it may represent the introduction of virus on contaminated fresh produce	Traceback implicated or suspected food items, look for possible related outbreaks linked to same source

feces (Lopman et al. 2012). However, the source of contamination for widely distributed outbreaks associated with leafy green vegetables and fresh or frozen berries has not been adequately characterized (Kokkinos et al. 2012; Maunula et al. 2013). A recent study of berry production in Europe evaluated 785 samples collected from four countries over 2 years (Maunula et al. 2013); human adenovirus was found in 9.5 % of irrigation water samples and from up to 3.2 % of berries at retail. Norovirus GII was detected in 3.6 % of irrigation water samples but not at processing or in the berries at retail (Maunula et al. 2013). The presence of human adenovirus across the

production and distribution systems demonstrates the potential for irrigation water to contaminate fresh produce. More thorough investigation of outbreaks associated with fresh produce is needed to establish the public health importance of this transmission pathway.

Water used for irrigation of fresh produce items is not usually treated to drinking water standards (Steele and Odumeru 2004). Surface water supplies may be contaminated from inadequately treated sewage discharges, non-point source run-off or recreational use. Ground water supplies may be contaminated from septic system failures (Borchardt et al. 2011). Contaminated irrigation water applied to plant materials may result in attachment of virus particles, which may remain viable throughout the distribution and shelf-life of the food product (El-Senousy et al. 2013). A field study demonstrated the presence of norovirus GI on strawberries from 6 of 16 plots 1 h after irrigation, even though no norovirus was identified in the irrigation water (Brassard et al. 2012). Only one of the six plots was positive for norovirus before irrigation. Green onions and leafy green vegetables can take up virus particles through root hairs and incorporate them into plant tissues (Chancellor et al. 2006; Hirneisen and Kniel 2013). However, this has been demonstrated more efficiently for hydroponic systems than field-grown crops.

Beyond the use of water for irrigation, the water used in applying pesticides and in processing of harvested produce is possible source of contamination (Verhaelen et al. 2013). Because these applications of water involve more direct contact with plant materials than those designed to deliver water primarily to roots, they represent important potential sources of contamination. Distinguishing the potential roles of irrigation or processing waters in contamination of fresh produce items in an outbreak investigation presents inherent difficulties, particularly when investigations are conducted weeks to months after the outbreak's occurrence. In several outbreaks traced to contaminated strawberries from China and lettuce from France, the occurrence of multiple norovirus strains and enterotoxigenic *Escherichia coli* strongly suggests sources of contamination from sewage rather than from individual food handlers (Ethelberg et al. 2010; Mäde et al. 2013). Limited surveys of fresh produce items, including bagged pre-washed salad mixes, have demonstrated the presence of noroviruses in a small percentage of products available at retail (Baert et al. 2011; Mattison et al. 2010). However, these have not generally been linked to foodborne disease outbreaks.

5. ROLE OF SURFACE CONTAMINATION

Transmission of viruses between hands, environmental surfaces, and food has been extensively studied using surrogates and pathogenic viruses (Bidawid et al. 2004; D'Souza et al. 2006). These studies have been useful for establishing quantitative data on virus transfer that can be used in risk assessments and

to design intervention strategies to reduce norovirus transmission in food service settings (Escudero et al. 2012; Shieh et al. 2014). From these studies it is clear that norovirus can be transferred from contaminated hands to work surfaces, utensils and food, and from contaminated food to utensils, work surfaces, and hands (Grove et al. 2015). The efficiency of virus transfer depends on the type of material and whether conditions are wet or dry (Sharps et al. 2012). Across all of these studies it is clear that the most important environmental surface leading to transmission of norovirus is the hands of food handlers (Boxman et al. 2008; Rönnqvist et al. 2014).

A year-long survey of norovirus prevalence in food service establishments in the Netherlands demonstrated the presence of norovirus in environmental samples collected throughout the year (Boxman et al. 2011). Approximately 1 % of kitchen samples and 3 % of employee bathroom samples were positive for norovirus in the absence of any evidence of foodborne transmission. During outbreak investigations, 29 % of kitchen samples and 53 % of bathroom samples were positive for norovirus (Boxman et al. 2011). Norovirus strains detected in the prevalence study reflected the distribution of viruses in the community, and were more likely to be detected during winter months. Thus, during this study period, transmission of noroviruses in the community appeared to be the primary source of virus contamination within the food service establishment, with amplification by infected food handlers during outbreaks.

In institutional settings, where resident populations have many opportunities for repeated exposures to contaminated environmental surfaces, environmental contamination contributes to protracted transmission over time (Table 5.1). In most food service settings, the transient customer base limits the opportunities for transmission by environmental contamination. However, common exposure to a contaminated bathroom led to a point source outbreak that initially appeared to be a point source foodborne outbreak (Repp et al. 2013). Another point source outbreak was associated with handling or eating food that was carried in a reusable grocery bag that had been stored in a bathroom used by a person with norovirus-like illness (Repp and Keene 2012).

The role of surface contamination in foodborne transmission of norovirus is difficult to establish in the context of outbreaks in which infected food handlers are present and provide a direct route for contaminating food. Complex transmission dynamics involving multiple infected food handlers and contaminated work surfaces and utensils all likely contribute to amplification of the original source of virus introduction. Thus, controlling outbreaks in food service establishments requires a multifaceted approach with a focus on identifying infected food workers, disposing of foods they may have handled, and cleaning and sanitizing environmental surfaces they may have contaminated. Although the focus of this attention is usually placed on food contact surfaces, bathroom facilities used by food handlers also warrant enhanced consideration.

6. SUMMARY AND CONCLUSIONS

The development and dissemination of norovirus genotype and sequence-based surveillance is changing the approach of public health agencies to the investigation and control of norovirus outbreaks. Rather than always taking the default approach that foodborne norovirus outbreaks are the local manifestation of illness among food handlers, clusters of restaurant outbreaks will be increasingly investigated as separate nodes in broader outbreaks due to distribution of contaminated produce items. The genotype and sequence-based investigation of norovirus outbreak clusters represents a convergence of surveillance methods between viral and bacterial foodborne pathogens. Harmonizing approaches to cluster-based investigations should provide motivation to enhance surveillance activities at all levels of the public health system.

The importance of controlling environmental contamination during production has always been very clear for shellfish. The potential role of irrigation water as a source for norovirus remains to be fully established, but it could be significant. Foodborne outbreaks occurring out of the normal season for peak person-to-person spread should always be investigated with this hypothesis in mind. When separate establishment-based outbreaks are linked by genotype sequence, investigations to identify a common source should be aggressively pursued. Surveillance activities that are integrated at national and international levels provide a framework for fundamentally improving our understanding of the dynamics of norovirus transmission.

Foodborne norovirus outbreaks may be initiated from infected food handlers, contaminated foods, or contaminated environmental surfaces, in decreasing order of importance. Controlling foodborne norovirus outbreaks in food service establishments requires a multifaceted approach with a focus on identifying and excluding infected food workers, disposing of foods they may have handled and cleaning and sanitizing environmental surfaces they may have contaminated.

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CHAPTER 6

Case Studies and Outbreaks: Fresh Produce

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1. INTRODUCTION

The number of foodborne outbreaks linked to the consumption of fresh produce has been on the rise (Gould et al. 2013). Over 45 % of outbreaks of known etiology reported to the Centers for Disease Control and Prevention (CDC) between 1998 and 2008 were caused by foodborne viruses, especially human norovirus (HuNoV) and to a lesser extent hepatitis A virus (HAV) (Gould et al. 2013). In one recent study, a majority of viral outbreaks were attributed to the consumption of leafy vegetables (35 %) or fruits and nuts (15 %) (Painter et al. 2013). Contamination of fresh produce may occur prior to distribution in the wholesale or retail market through contaminated irrigation water, poor hygiene of harvesters, and/or during the processing and packaging steps due to improperly sanitized equipment or wash water. Contamination during production and processing has recently led to large scale outbreaks of HuNoV associated with strawberries in 2012 (Bernard et al. 2014; Made et al. 2013) and of HAV in green onions in 2003 (Wheeler et al. 2005).

The health risk from consumption of contaminated produce is difficult to quantify as few surveillance studies have been published to date on either HAV or HuNoV. In one large scale study, HuNoV genome was detected by real-time quantitative RT-PCR in 25 % (216/850) of tested produce from three countries (Baert et al. 2011); 653 leafy greens, 179 soft berries, and 18 other items were tested. However, sequence confirmation was unsuccessful for a majority of samples that tested positive, and infections or outbreaks from consumption of these products were rarely reported. Although positive genomic detection may indicate past contact with HuNoV in the produce production chain, it is difficult to ascertain the actual risk to consumers as it is not yet possible to determine the infectivity of the detected virus. Similar challenges exist for interpretation of surveillance data on HAV as infectivity is difficult to determine due to limited culturability of wild-type virus. As such, the focus remains on preventative measures including good agricultural practices and personal hygiene to avoid contamination. In the event of an outbreak of illness, rapid reporting, epidemiological investigation, and sensitive virus detection methods in the suspected food vehicle are needed to determine the source of contamination and to prevent any further contaminated products from entering into the market.

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Foodborne virus outbreaks may also result from poor hygiene in the kitchen and improper food handling by the staff. Salads and vegetables, which are often minimally processed and consumed raw, pose a high risk. Education on food hygiene for all personnel involved in food handling, adherence to hand washing recommendations, and implementation of policies to minimize the presence of ill kitchen staff are crucial in the prevention of such disease outbreaks. The case studies presented here highlight some of the successes and difficulties involved in identifying the cause of foodborne disease outbreaks and implementing appropriate control measures to prevent further spread of illness. Detection of foodborne viruses in suspected foods remains challenging due to the presumed low viral copy numbers, the complex and diverse nature of food matrices, and the difficulty in tracing back the actual outbreak samples for testing. Indeed, in HuNoV outbreak investigations between 1998 and 2008, epidemiological data (55 %) and other supporting information (30 %) were primarily used to identify the source of contamination; in only 2 % of the cases was HuNoV actually isolated from a food sample (Hall et al. 2012).

The outbreaks presented in this chapter have been selected as key examples, where the virus transmission route was either clearly identified or highly presumed due to strong epidemiological evidence. The primary aim of this chapter is to help elucidate causes of past outbreaks in order to formulate new policies to prevent similar outbreaks in the future. Outbreaks covered in this chapter focus on HuNoV (Table 6.1) and HAV (Table 6.2), two of the most epidemiologically significant foodborne viruses, and span the time frame from 1990 to 2013.

Table 6.1 Summary of produce-related norovirus outbreaks described in this chapter

Implicated produce item	*Suspected route of contamination*	*Cases*	*Country*	*Year*	*Reference*
Strawberries	Production/ Processing	~11,000	Germany	2012	Bernard et al., (2014), Made et al. 2013
Raspberries	Production/ Processing	>1000	Denmark	2005	Falkenhorst et al. (2005), Korsager et al. (2005)
	Production/ Processing	~200	Finland	2009	Maunula et al. (2009)
	Production/ Processing	433	Sweden	2006	Hjertqvist et al. (2006)
	Production/ Processing	75	France	2005	Cotterelle et al. (2005)
	Production/ Processing	30	Sweden	2001	Le Guyader et al. (2004)
Lettuce	Production/ Processing	260	Denmark	2010	Ethelberg et al. (2010)
Salad or raw vegetables	Food handler	147	France	2011	Mayet et al. (2011)
	Food handler	101	Germany	2009	Wadl et al. (2010)
	Food handler	159	Israel	1999	Grotto et al. (2004)
	Food handler	182	Austria	2006	Schmid et al. (2007)

Table 6.2 Summary of produce-related hepatitis A virus outbreaks described in this chapter

Implicated produce item	*Suspected route of contamination*[a]	*Cases*	*Country*	*Year*	*Reference*
Strawberries	Production/Processing	213	USA	1998	Hutin et al. (1999)
Strawberries	Production/Processing	~1400	USA	1990	Niu et al. (1992)
Pomegranates		>150	USA	2013	CDC (2013)
Blueberries		81	New Zealand	2002	Calder et al. (2003)
Berry blend			Several European nations	2013	EFSA (2013)
Berries		71	Denmark, Sweden, Norway, Finland	2012–2013	Lassen et al. (2013)
Green onions	Production/Processing	43	U.S.A.	1998	Dentinger et al. (2001)
Green onions	Production/Processing	600	U.S.A.	2003	Wheeler et al. (2005)
Orange juice		351	Nine European nations	2004	Frank et al. (2007)
Semi-dried tomatoes		>200	Australia	2009	Anonymous (2009)
Semi-dried tomatoes		59	France	2010	Gallot et al. (2011)
Semi-dried tomatoes		13	Netherlands	2009–2010	Petrignani et al. (2010)
Leafy greens	Food handler	16	Canada	2005	Heywood et al. (2007)

[a]Unless specified, the source of contamination is not clearly reported

2. CASE STUDIES AND OUTBREAKS

2.1. Norovirus Outbreaks

Human norovirus is the leading cause of foodborne disease outbreaks in the United States, with leafy greens and fruits accounting for over half of outbreaks attributable to a single commodity (Hall et al. 2012). In addition, fresh produce is often contained in complex food products that are commonly implicated in HuNoV outbreaks, such as sandwiches and salads. Although a majority of outbreaks from 1998 to 2008 were suspected to be linked to contamination by food handlers (82 %) (Hall et al. 2012), there have been several reports of outbreaks resulting from produce contaminated prior to food handling, especially raspberries (Falkenhorst et al. 2005; Hjertqvist et al. 2006; Korsager et al. 2005; Maunula et al. 2009). The percentage of produce contamination occurring in the production and processing phase is likely underestimated, with approximately 50 % of the outbreaks having an unidentified source of contamination (Hall et al. 2012).

One of the largest recorded outbreaks in recent history occurred in various schools and childcare establishments in eastern Germany in the fall of 2012 and was associated with the consumption of frozen strawberries (Bernard et al. 2014; Made et al. 2013). Approximately 11,000 people were affected, including 38 cases of hospitalizations. Two case studies indicated that consumption of strawberry compote was most associated with illness, with an odds ratio (OR) of 8.20 in one study and 16.84 in another (Bernard et al. 2014). A web-based survey identified strawberry quark (OR = 27.13) in the first wave, strawberry compote (OR = 33.80) in the second wave, and strawberry quark (OR = 45.42) in the third wave as the likely culprits. Finally, in an e-mail survey, strawberry compote had a significant positive association with illness (OR = 30.61). The epidemiological data strongly suggested that strawberries were responsible for these outbreaks with four independent studies reaching the same conclusion. Trace-back investigations revealed that all caterers implicated in the outbreaks had received frozen strawberries from one large lot imported from China. A recall was put in place to remove over 11 tons of the implicated lot to prevent further outbreaks (Bernard et al. 2014).

Samples of frozen strawberries from the suspected lots were tested for the presence of HuNoV (Made et al. 2013). Seven of 11 samples from three different canteens and four original boxes from the warehouse tested positive for HuNoV GI and GII. Only RNA extracted from one subsample could be reliably genotyped, showing a recombinant GII.6 (polymerase)/II.13 (capsid) virus, a combination that had not been previously reported in Germany. This recombinant was also detected in stool samples from some of the patients sickened in this outbreak with a 99.5–100 % sequence identity in the ORF1/ORF2 junction. In addition, short sequences generated from the real-time RT-PCR products revealed other genotypes such as GI.9 and GII.6. both of which were also detected in some of the stool samples (Made et al. 2013). The detection of

multiple HuNoV strains in the strawberry lots suggested that contamination may have occurred due either to unclean irrigation water or multiple events in the harvest to processing chain rather than from a single infected food handler. These outbreaks have led to a new European Union (EU) regulation requiring 5 % of frozen strawberries imported from China into the EU to be screened for HuNoV (European Union 2012).

From May to September of 2005, there were a series of six HuNoV outbreaks in Denmark linked to frozen raspberries imported from Poland, affecting over 1000 individuals (Falkenhorst et al. 2005; Korsager et al. 2005). In the first of these outbreaks, approximately 450 patients and employees in two hospitals became ill. Case control studies of 120 employees and in-patients at the hospitals found that consumption of a dessert containing frozen raspberries was associated with an increased risk of disease (Korsager et al. 2005). The implicated batch of frozen raspberries was withdrawn from the market but delays in the implementation resulted in a second large outbreak among elderly patients receiving meals from a single caterer as part of a "meals on wheels" program. In this second outbreak, an estimated 400 people became ill with over 23 requiring hospitalization (Falkenhorst et al. 2005). Two smaller outbreaks of HuNoV were subsequently reported in August, suggesting that the removal of implicated raspberries had been incomplete.

In the last of these six outbreaks, frozen raspberries from a different Polish producer with a different Danish importer were implicated. A cohort study revealed that consumption of a cold dessert with raspberries in the company canteen was associated with illness with a risk ratio of 12.2 and an attack rate of 82 % (Falkenhorst et al. 2005). Testing of available stool samples associated with the initial lot of raspberries identified GII.4 in one outbreak, GII.b in another, and GII.7 in two of the other outbreaks. Within each of the investigated outbreaks, only a single strain of virus was identified. The presence of different strains in these outbreaks may be due to raspberries grown on several small scale farms in Poland that were combined to make a single large batch followed by being frozen and then exported to Denmark. Once again, the identification of different strains in these outbreaks suggests that the contamination was not likely to be due to a single food handler. This series of outbreaks highlights the importance of rapidly identifying the suspected food item, efficiently removing it from the market, and communicating with food caterers regarding the risk to prevent further outbreaks from occurring.

Epidemiological studies of foodborne outbreaks in Finland (2009) (Maunula et al. 2009), Sweden (2006) (Hjertqvist et al. 2006), and France (Cotterelle et al. 2005) suggested that the consumption of uncooked, imported frozen raspberries used in a wide variety of dishes, was the likely cause. In the Finnish outbreak, a cohort study revealed that people consuming berries were three times more likely to develop illness than those who did not. Upon testing the remaining raspberry samples, three of five frozen samples tested positive for HuNoV GI by real-time PCR. Virus isolated from one food sample was identified as GI.4, matching the genotype found in the two available stool samples of

patients. Sequences within a short capsid region of the genome were identical between the patient and the berry sample (Maunula et al. 2009). In France, five of six stool samples from ill students were positive for HuNoV GI.5. However, despite strong epidemiological data implicating raspberries (relative risk (RR) = 3.3), an unopened package of raspberries from the same producer tested negative. It was believed that the raspberries were contaminated prior to handling by the kitchen staff as no illnesses were reported by the staff and no association was found between illness and consumption of other foods prepared by the same staff (Cotterelle et al. 2005). In Sweden, a single brand of raspberries imported from China by one distributor was determined to be the common source for a cluster of outbreaks (Hjertqvist et al. 2006).

In a different Swedish outbreak in 2001, stool samples from five of nine patients tested positive for HuNoV GI and sequencing showed that a single strain was likely responsible for the gastroenteritis (Le Guyader et al. 2004). When the same primer set was used, suspected raspberry samples tested negative, although a positive signal was obtained when a different set of primers was used. Upon further investigation, nested PCR with the outbreak specific primers yielded a positive fragment of expected size in raspberry samples. It was hypothesized that the strain responsible for the outbreak represented only a small percentage of HuNoV in the raspberry samples. Indeed, there have been reports where multiple strains have been detected in both the food sample and the patients. There may be yet unidentified factors which promote the replication of one strain over another in certain individuals. The laboratory results obtained in this investigation highlight some of the complexity and challenges involved with outbreak investigations, even when patient stools and suspected foods are available for direct testing. Due to the lack of sensitive laboratory methods for testing for norovirus contamination, food samples have tested negative for HuNoV despite strong epidemiological data (Cotterelle et al. 2005; Wadl et al. 2010).

In addition to soft berries, lettuce and salads are other commodities often implicated in HuNoV outbreaks. In January of 2010, a series of 11 gastroenteritis outbreaks with over 260 cases was reported in Denmark (Ethelberg et al. 2010). Ill persons in each outbreak had consumed catered sandwiches, and upon further investigation, lettuce was identified as the likely common food ingredient amongst these outbreaks. The caterers who supplied the meals all reported having purchased lollo bionda lettuce from one of two suppliers, who had in turn purchased the lettuce from a single wholesaler. Pooling the data from questionnaires for the three different types of sandwiches containing the suspected lettuce gave a relative risk of 6.2. Testing of lettuce collected from two of the catering companies revealed the presence of HuNoV genogroup II. Of the 25 stool samples tested, 12 tested positive for genogroup II, 2 for genogroup I and 9 had mixed infections. Early sequencing results showed the presence of at least three genotypes. Upon inquiries sent through the European Center for Disease Prevention and Control's network, three outbreaks in Norway were attributed to the same

lettuce type. It appeared that the same batch of lettuce that had caused disease in Denmark had been exported to Norway and was likely responsible for the outbreaks. This case study demonstrates the necessity for rapid dissemination of information both inside and outside the country, especially as produce is increasingly imported across borders. As the lettuce appears to have been contaminated by multiple infectious agents prior to distribution to the catering companies, it is likely that human fecal matter came into contact with the lettuce at the production and processing levels possibly through contaminated water.

Contamination contributed by food preparers is another major source of localized HuNoV outbreaks. Several HuNoV outbreaks associated with the consumption of raw produce have been reported in military units (Grotto et al. 2004; Mayet et al. 2011; Wadl et al. 2010). In 2011, over 100 people in a French military parachuting unit reported falling ill between the 12th and 13th of April (Mayet et al. 2011). Testing of 69 food items used in the four meals on the 11th and 12th of April, found a significant association between occurrence of illness and consumption of salad and raw vegetables (OR = 2.1 and OR = 2.1, respectively). Both items were prepared by a cook who had been mildly ill prior to the outbreak. Stool samples from the ill cook tested positive for HuNoV GI. Carrots and salad served at lunch and tomatoes served at dinner, were found to be positive for HuNoV GI. Amongst all the reported cases, 72 % had eaten at least one of these food items. Although no stool samples were collected from any of the individual cases, the presence of HuNoV GI in the symptomatic cook and in the food items he prepared, suggest that he was the likely source of the outbreak.

Outbreaks at military facilities often provide ideal scenarios for epidemiological case studies due to the closed nature of the facility, the common food items shared, and the existence of policies for sampling and storage of foods served. Raw salad was associated with a different HuNoV outbreak in a German military facility in the winter of 2009 (Wadl et al. 2010). Initial investigations revealed that 104 of 815 military base members fell ill with vomiting or diarrhea between December 24th 2008 and February 3rd 2009, with a peak of 49 between January 7th and 9th. To determine whether food served in the canteen was involved in the outbreak, a standardized questionnaire was administered to 247 members of the headquarters company, asking such information as the date and time of symptom onset and possible exposures, including food consumption and contact with ill persons. Eating salad from a self-service salad buffet was associated with a higher risk of disease on both the 6th and 7th and the risk of disease increased with the number of days on which the salad was consumed. Within the entire cohort and amongst those who had eaten lunch on the 6th and 7th, consumers of salad had a 4.4–4.8 times greater risk of becoming ill compared to non-salad consumers.

Analysis of stool samples from sick individuals revealed that approximately 28 % were positive for HuNoV GII by RT-PCR, with five randomly selected samples having identical nucleotide sequences in the polymerase and the ORF

1/2 region. Of the canteen employees testing positive for HuNoV, two were identified to have been working on the 6th and/or 7th of January. One employee reported having nausea on the 5th but continued to work through the 6th. The other reported no symptoms but had cleaned the salad bar on the 6th and 7th. Sequencing results from these two individuals revealed the same GII.4 2006b subtype as present in the patients with only 1.4–2.4 % difference between their sequences. Due to these slight sequence differences, it is not clear whether the canteen employees were the source of contamination for the salad. However, it is possible that the two HuNoV-positive canteen employees had been shedding the outbreak strain at the time the samples were collected (1–4 weeks after the start of the outbreak). It has been previously reported that infected persons can shed several different HuNoV strains at the same time. None of the 33 tested food items, including salad and dressing, was positive for HuNoV. To control the outbreak, ill individuals were isolated or sent on sick leave for 1 week, while healthy persons were sent home for 2 days to reduce virus transmission. Cleaning and disinfection of the canteen was done using virucidal agents, and canteen employees with positive stool samples were excluded from work until they tested negative. Such preventive measures likely minimized the scale of this outbreak.

In an Israeli military facility, there was an outbreak of acute gastroenteritis involving 159 soldiers between December 20th and 21st, 1999 (Grotto et al. 2004). A case control study of exposure to different food items served up to 48 h prior to the outbreak was conducted with 40 randomly selected cases and 44 controls. A high association of illness was found with the consumption of a fresh vegetable salad served at lunch on December 20th in the mess hall (OR = 4.38). Consumption of a fresh vegetable salad at breakfast on Dec 20th and 21st was also associated with illness (OR = 2.62 and OR = 2.86, respectively).

No food handler present during the outbreak reported being ill; however, one reported having been ill and vomiting 2 days before the outbreak but did not exclude himself from work. Inspection of the kitchen facility noted poor personal hygiene of the food handler responsible for salad preparation as well as the existence of potential cross-contamination between ready-to-eat salads and raw non-disinfected vegetables. Post defecation perianal swabs from all 3 non-ill food handlers tested negative and 4 of the 24 ill military personnel tested positive for HuNoV by RT-PCR. Samples from the food handler who had reported being previously ill were unavailable for testing. Although suspected foods were available for testing, only bacteriological analyses were performed, yielding negative results (Grotto et al. 2004). The availability of standardized, sensitive methods for detection of viral agents in foods will likely promote adoption of virus testing protocols in suspected viral outbreaks. The epidemiological data and inspection reports strongly indicated that the outbreak was likely due to contamination of raw vegetables and salad in the kitchen. The prompt closure of the kitchen and the separation of ill and healthy individuals likely decreased any secondary propagation of the outbreak.

In January 2006, a cluster of gastroenteritis cases with clinical symptoms of HuNoV infection was reported in Austria (Schmid et al. 2007). The outbreak lasted from January 17th to 24th with a peak on the evening of Jan 19th. Of the 182 individuals who fell ill, all except 10 had eaten at the company canteen within 2 days prior to disease onset. Epidemiological data indicated that consumption of salad on January 18th was associated with the highest risk of illness (RR = 2.82). Further investigation revealed that the most likely source of contamination was a kitchen assistant who had fallen ill with symptoms of gastroenteritis early on the 18th, but had continued working and had manually prepared the salad. In addition, kitchen inspection revealed poor hygienic conditions, such as the lack of a functional hand washing facility inside the kitchen area. Stool specimens from the kitchen worker tested positive for HuNoV by RT-PCR and sequencing of HuNoV from two of the cases identified the strain as GII.7. Food samples tested negative for bacterial pathogens and were not tested for the presence of HuNoV. To prevent future outbreaks at the facility, the kitchen was closed, the kitchen and canteen were disinfected, functioning hand wash facilities were requested, and a refresher training course on food hygiene was installed for the kitchen staff before being admitted back to work.

Several measures need be adopted in order to prevent outbreaks involving contamination via ill food handlers as described above. Firstly, anyone with gastrointestinal symptoms should immediately be removed from food handling areas. Policies should be in place so that employees who do not work through illness should have no fear of losing employment. Secondly, proper food hygiene education must be provided and reinforced for kitchen staff. HuNoV shedders may be asymptomatic or continue to shed virus for >3 weeks after symptoms have subsided (Atmar et al. 2008), so it is crucial that enforcement of protective measures such as hand washing and disinfection prior to handling food especially after usage of restrooms are followed. Finally, it is important to maintain proper facilities in the kitchen to aid in the maintenance of hygienic conditions.

2.2. Hepatitis A Virus Outbreaks

Since the introduction of vaccination programs in the United States in 1996, the overall frequency of HAV has declined and incidents have become more sporadic in occurrence. In Europe, the HAV notification rate decreased from 14.0 in 1997 to 2.6 per 100,000 in 2010 (European Centre for Disease Prevention and Control 2013). Although the overall number of HAV infections appears to be on the decline, produce-associated outbreaks of HAV have been increasingly reported. It has been estimated that there are greater than 1500 domestically acquired cases of foodborne HAV in the United States (Scallan et al. 2011); thus, HAV remains a significant public health threat.

One of the earliest reported outbreaks of HAV implicating a produce item occurred in 1998, sickening a total of 213 people in the states of Michigan and Maine, USA (Hutin et al. 1999). The illness was associated with the consumption of frozen strawberries in case–control studies conducted in these states

(OR = 8.3 and 3.4 in Michigan and Maine, respectively). The same lots of strawberries had been sent to 13 other states; however, only a small number of patients from four additional states (Arizona, Louisiana, Tennessee and Wisconsin) were found to have the same genetic sequence (VP3–VP1 and VP1–P2A junctions) as the virus isolated from 126 patients in Michigan and Maine. All of the infected individuals had consumed products containing strawberries from the same provider. The relatively limited spread of the outbreak indicated that contamination had most likely occurred at a low level and was not uniform. The implicated strawberries were grown in Mexico, processed in California, and shipped to school cafeterias to be served either in cups or incorporated into shortcakes. The lots of strawberries that were sent to Michigan were later withdrawn from the market.

An epidemiological study revealed that the fruit pickers had not been wearing gloves and had removed the strawberry stems with their fingernails. The same investigation indicated that the strawberries were likely contaminated prior to distribution, as the sealed containers were not opened prior to being used in various school kitchens. The genetic identity of HAV sequences from Michigan patients confirmed the epidemiological evidence that the outbreak originated from a single common source. During the time of packaging there were no records of any sick employees and there was minimal contact of the fruit with human hands. It was also unlikely that contamination occurred during the short time the fruit was held in the fields and transported for processing. In the processing plant, workers did not handle the fruit, and fecal contamination of the water used to wash the fruit or to prepare the sucrose slurry was unlikely, as coliform bacteria were not detected in the final product. Thus, it was concluded that contamination most likely occurred during harvest due to poor toilet and hand-washing facilities in the fields along with the hand contact required to remove the stem from each berry. With sequencing analysis, it was possible to link the apparently sporadic cases occurring in other states to the main outbreak in Michigan. Because the fruits implicated in this outbreak had been picked and processed approximately 1 year prior, it was not possible to clearly identify the exact source or the time at which contamination occurred. However, it has been previously shown that HAV remains infectious even after prolonged storage at low temperatures; indeed, HAV has been shown to survive for up to 5 weeks at refrigeration temperatures in packaged spinach (Shieh et al. 2009). If contamination of the strawberries had occurred in the field, HAV likely survived during refrigeration and remained potentially infectious when the product was consumed raw.

Berries have been frequently associated with HAV outbreaks. One hypothesis is that, similar to other soft fruits, berries require individual picking and that their fragile, soft surface can be easily damaged, allowing for possible virus internalization. Although internalization has not yet been documented with foodborne viruses, it has been suggested that damaged vegetable surfaces can harbor microorganisms and likely promote virus internalization, making it harder for them to be removed later via washing/elution.

Another outbreak involving berries was recorded in 1990 in a school in Georgia (USA), involving almost 900 people. This outbreak later spread to an institution for the developmentally disabled in Montana, where more than 500 became ill (Niu et al. 1992). A cohort analysis in the Georgia school identified strawberries in a shortcake as the source of infection (RR = 7.6) and a similar analysis suggested uncooked strawberries in desserts as the most likely source of transmission in Montana. The strawberries were harvested, processed, and packed the same day in a plant in California in 1988 and kept frozen until nationwide distribution in the fall of the following year. The processing company identified the implicated lot and withdrew the rest of the containers handled during that shift. A 350 base pair (bp) region of the viral capsid from both outbreak strains was identical. The contamination was believed to have occurred prior to processing and serving, although HAV was never detected in any of the lots of strawberries tested.

One of the most recent and largest outbreaks of HAV occurred in late May of 2013 in Colorado and spread to ten more states. This outbreak involved more than 150 people with 44 % of them being hospitalized. Epidemiological investigation identified pomegranate arils from a frozen berries mix to be the suspected food source. Testing of a majority of the infected individuals led to the conclusion that HAV genotype 1B was the responsible agent. Suspect products were recalled and further shipment from the same company was detained. The identified genotype is not usually found in the US but is endemic in Middle Eastern countries. The frozen berries mix contained fruits originating from four different countries and the pomegranate seeds were shipped from Turkey, where genotype 1B is prevalent. Although several food samples were collected and tested for HAV contamination, there was no confirmed detection of the virus. More specific information on the source of contamination is not available but it was speculated that irrigation water or an infected food handler came in contact with that particular batch of pomegranate arils (Centers for Disease Control and Prevention 2013).

The HAV has been recognized as a causative agent of other outbreaks associated with berries on a world-wide scale, with one occurring in New Zealand between January and May of 2002 (Calder et al. 2003). A case control study of 81 confirmed cases identified raw blueberries as the implicated vehicle (OR = 7.6) and a trace-back investigations found the existence of multiple opportunities for contamination before, during, and after harvest. Virological investigation led to the detection of HAV not only in patients' samples but also in the blueberries. It was confirmed to be the same strain with more than 92 % similarity in a 170 bp fragment of the VP3 capsid region.

In January of 2013, there was a multi-country outbreak of HAV in several European countries including Germany, the Netherlands and Poland, with a total of 15 confirmed cases. All infected individuals had returned from a trip to Italy (European Food Safety Authority and European Center for Disease Prevention and Control 2013). During the same time, there was an increase in the number of HAV infections in northern Italy and Ireland, suggesting a con-

tinuous common source of infection. The suspected vehicle was a blend of frozen berries; the virus was successfully isolated from a package that one of the infected patients had partially consumed. Seven sequences of HAV genotype 1A isolated from cases in different countries (the Netherlands, Germany and Italy) and in different laboratories showed a 100 % similarity indicating that the cluster of HAV infection was from a common source.

Another outbreak of HAV originated in Denmark, which later spread to Sweden, Norway and Finland (Lassen et al. 2013). Between October 2012 and July 2013, there were 71 confirmed cases of HAV infection in these four countries. Epidemiological investigations suggested that frozen berries (mostly strawberries) were the suspected food vehicle (OR = 12.5 in Danish case control study). Genotype 1B of HAV was identified from all four countries, which was later more specifically characterized as three sequences with a maximum 2 % diversity. The four Nordic countries recommended boiling all frozen berries before consumption. Through the European HAVNET network and other available information, Egypt was indicated as the most likely country of origin of the berries. Samples of frozen berries were taken from the freezers of HAV patients in all affected countries but no virus was detected. As a result, it was not clear if the source of infection for the multi-country outbreaks was the same or not. During the outbreak, the European CDC notified other European countries of these outbreaks. Following the two described outbreaks in Europe since January 2013, 1315 cases of HAV infection were discovered in 11 European countries (Italy, France, Germany, Poland, Ireland, Netherlands, UK and the four Nordic countries) of which 240 shared the same strain sequence. However, there is no solid evidence to date that links all of these cases to a single contamination source.

One of the most highly recognized produce item linked with HAV outbreaks is green onions. Several outbreaks of HAV caused by contaminated green onions have been reported, including one taking place in 1998 in a restaurant in Ohio (Dentinger et al. 2001). A matched case–control study identified a menu item containing green onions as the likely vehicle of illness (OR = 12.7). All restaurant employees who worked during the time of the outbreak tested negative for HAV, while sequences from serum samples of patients were identical, indicating a common source of infection. When contamination occurs at a restaurant, an ill food handler is often suspected; however, more menu items would likely have been contaminated through the extensive food handling that often takes place in such a food service establishment. Since this was not the case in this outbreak, contamination was believed to have occurred from a single source and most likely at the pre-harvest or harvest stage where many opportunities for contamination exist due to improper application of good agricultural practices.

A highly publicized HAV outbreak associated with produce occurred in a restaurant in Pennsylvania in 2003 (Wheeler et al. 2005); 600 individuals became ill, over 124 were hospitalized, and three died. All restaurant workers were tested but none was HAV-positive. Sequences of HAV from 170 ill patients were identical. Uncooked green onions prepared in a mild salsa dish

and served to all customers was the ingredient most associated with illness (OR = 33.3). An FDA trace-back investigation indicated that two farms in northern Mexico were the source of the green onions. The contamination of green onions was hypothesized to have occurred at the farms either by contact with workers (or workers' children) that were infected with HAV during harvesting or packing or through contact with contaminated water used for irrigation, rinsing, cooling or icing of the product. After leaving the farms, onions were not re-packed or re-iced. A month after the onset of the outbreak, FDA issued a consumer alert and imposed an import ban on farms that had supplied the green onions. From molecular epidemiologic investigations, it was possible to link this outbreak with three other sporadic occurrences that had previously been reported in restaurants in Tennessee, North Carolina, and Georgia. The strains identified in patients' sera had nearly identical sequences (less than 0.3 % diversity) and were similar to at least one sequence of HAV isolated from infected individuals in Mexico. No clear relationship between these outbreaks could initially be inferred but it was proposed that all originated from closely related HAV strains circulating in northern Mexico. Later, whole-genome sequencing of HAV isolates confirmed that there were a number of heterogeneous strains contaminating the green onions, suggesting a common source of infection for all four outbreaks (Vaughan et al. 2014).

These outbreaks highlight several produce-specific characteristics and preparation practices that can allow cross contamination and spread of foodborne viruses. Initially, the green onions on the farm required extensive handling during harvesting and preparation for packing, including the removal of outer skins. If any of the workers had not practiced good hygiene during harvest, he/she could have easily contaminated the produce. The liquid on the surface of onions from either rinsing or from melting ice may also have facilitated HAV transfer between contaminated and non-contaminated onions. Once inside the restaurant kitchen, the green onions were rinsed with tap water, roots cut, rubber band around the bundle removed, and chopped with the use of an electric dicer. The use of this electric dicer for processing of all batches may have contributed to cross contamination of the initially non-contaminated batches. Similarly, inadequate cleaning of utensils and equipment used for food preparation may have promoted virus transfer.

Dates, orange juice, and sundried tomatoes have also been implicated in foodborne disease outbreaks. This may be attributed to the increased consumption of foods of international origin. Also, more elaborate and thorough epidemiological and diagnostic tools are now available to recognize outbreaks associated with foods. In a recent surveillance study, HAV was detected by RT-PCR in 2 of 185 batches of dates in the Netherlands (Boxman et al. 2012). In 2004, a major outbreak of HAV among tourists returning from Egypt involved 351 case-patients from 9 European countries that were all infected with a single strain (genotype 1B) of HAV (Frank et al. 2007). Unpasteurized orange juice consumed at a specific hotel was identified as the vehicle of infection. This outbreak may have been averted if the travelers were aware of HAV endemicity in Egypt and had been vaccinated prior to travel.

Semi-dried tomatoes (sun-dried tomatoes) were implicated in an outbreak involving more than 200 cases of HAV in Australia in late 2009 (Anonymous 2009). The virus was successfully detected in the suspected food samples as well as in the clinical samples from infected individuals. Frozen, semi-dried tomatoes were likewise identified as the vehicle of infection (age-adjusted OR = 8.5) in all primary HAV infection cases in another outbreak involving 59 patients in France during January 2010 (Gallot et al. 2011). A trace-back investigation identified a single supplier in France who had imported frozen semidried tomatoes from Turkey and had then subsequently supplied the tomatoes to the three sandwich shop chains involved in the outbreak. The semi-dried frozen tomatoes had been thawed followed by the addition of oil and herbs without any heating or other inactivation steps before consumption. However, this batch was no longer available at either the supplier or at the sandwich shops for testing or for recall. During the same time period (late 2009-early 2010), 13 other HAV cases were reported in the Netherlands (Petrignani et al. 2010). A case–control study once again identified semi-dried tomatoes (OR = 20.0) as the vehicle of transmission, although the virus could not be detected in any of the 81 food samples tested.

All of these outbreaks were caused by highly similar IB strains. The French outbreak strain differed only by 2 and 3 nucleotides as compared to the Australian strain (based on a 300 bp fragment of the VP1–2A region) and the Dutch strain (based on a 430 bp fragment), respectively. The Dutch strain was found to be identical to that found in patients involved in the outbreak in Australia (based on a fragment of the VP1-2A region). Surprisingly, a year later in October 2011, there were two more cases of HAV with 100 % strain identity (based on 505 bp of the VP1-2PA region) to the Dutch outbreak strain of the prior year and another two with over 90 % sequence similarity with a strain associated with the consumption of semi-dried tomatoes in England (Carvalho et al. 2012). One month later, another cluster of five HAV cases were reported in the Netherlands (Fournet et al. 2012). Two of the strains were identical to the ones implicated in the earlier HAV outbreak in the Netherlands during the first half of 2010, while one was closely related to the sequence found in a strain during the earlier 2009 outbreak in Australia and another strain that had caused an outbreak in France in 2010. Despite epidemiological investigations implicating salads, including those containing semi-dried tomatoes, no common source could be identified. It was hypothesized that this outbreak could potentially be linked to the British one and that all of the earlier outbreaks could have shared, as a common source, semi-dried tomatoes contaminated with multiple strains of HAV.

Apart from indirect productivity losses, the outbreaks can have high direct medical costs associated with them. For instance, in 2005 there was a symptomatic laboratory-confirmed case of acute hepatitis A reported to health authorities in Canada (Heywood et al. 2007). The patient worked as a food handler in a restaurant preparing ready-to-eat (RTE) foods, including leafy greens. In total, 16 laboratory confirmed cases of hepatitis A were reported among

patrons who had consumed food at this restaurant. To manage the outbreak and protect the community from further exposure to the virus, the public health authorities offered HAV vaccine prophylactically to 750 of the patrons and restaurant staff and an additional 210 people that were closely affiliated to the reported cases. In addition, a hotline was installed to disseminate pertinent information, and seminars on safe food handling practices were offered to the restaurant staff. Food samples were not available for testing but it was concluded that a food handler was infected on a trip to a country with high HAV endemicity and his colleagues at the restaurant were found to display poor personal hygiene practices.

3. SUMMARY AND CONCLUSIONS

There are several factors that contribute to the current inability to accurately determine the source of infection in many outbreak investigations. This leads to underreporting and underestimation of the true number of foodborne viral infections. In HAV outbreaks in particular, identifying the possible source of infection may be more challenging as the virus incubation period can be as long as 6 weeks, making accurate recall of the suspected product more difficult. Additionally, as evidenced in some of the outbreaks, the extended shelf life of produce that are packaged and kept at freezing temperatures complicates the investigation on the origin of contamination.

The lack of sensitive methods to recover foodborne viruses from the diverse nature of implicated produce items also contributes to the high percentage of outbreaks with unidentified sources. Few robust and sensitive methods to detect HAV or HuNoV in produce commodities are available and even fewer methods have been validated on an international level (Stals et al. 2013). Even if epidemiological evidence implicates a certain type of produce as the likely vehicle of infection, only part of a given produce lot may actually be contaminated. Thus, the detection of foodborne viruses in the implicated produce may fail merely because of inadequate and unrepresentative sampling. This complicates the selection of food samples to be analyzed in the laboratory, even if the suspected food items are still available for testing. Indeed, few methods have been successfully utilized in outbreak investigations, where contamination levels were suspected to be low and not always uniform. In addition, the samples are subject to deterioration because of non-ideal conditions during collection, transfer and storage prior to testing.

Identifying the source of contamination may further be hindered by the geographic distribution of contaminated items as cases can be disseminated across wide geographical areas (ie, multiple European countries or several US States). Through internationalization of the market, it has become a common practice to consume produce in a country different from where it was originally produced or packaged. For example, from 1998 to 2007, imported produce accounted for as many as 23 % of outbreaks traced to FDA-regulated produce

while domestic products accounted for 46 % of outbreaks during the same time period (Food and Drug Administration 2007).

To advance our understanding of the epidemiology of these viruses, some critical questions need to be addressed, such as the frequency and stages of the farm-to-fork continuum where contamination may likely occur. As we learn more about the characteristics, transmission routes and incidence of HAV and HuNoV in produce items, better policies should be developed to prevent and control these outbreaks in the future. A recent study was undertaken amongst four European countries (Czech Republic, Finland, Poland and Serbia) to monitor the potential of enteric viruses to enter the berries production chain (Maunula et al. 2013). Samples taken during the production, processing, and at point-of-sale were tested for HAV and HuNoV GI and GII by RT-PCR; HuNoV GII was detected in two (3.6 %) irrigation water samples at the berry production stage with an average estimated concentration of 1.1×10^3 PDU per 1 L of water. However, neither HuNoV nor HAV was detected at the point-of-sale location (Maunula et al. 2013).

In addition to underreporting, the presence of asymptomatic shedders may also obscure the true incidence of foodborne infection. The period during which an infected individual who may be involved in the harvesting, processing, or serving of produce items can shed infectious virus in the feces without exhibiting any symptoms, remains unknown (Patterson et al. 1993). In highly endemic areas, HAV mostly develops asymptomatically among young children under 5 years of age (Pinto et al. 2010). Further complicating the issue of virus transmission and outbreak propagation is evidence of pre-symptomatic fecal excretion primarily by HuNoV-infected individuals during the incubation phase of the disease (Lo et al. 1994). Another feature complicating the investigation of foodborne outbreaks, especially with HuNoV, is the difficulty in differentiating person-to-person spread versus a true food-borne transmission (Verhoef et al. 2010). As a result, it may be difficult to identify the suspected produce item.

Another feature highlighted through outbreak investigations is the significant contribution of a surveillance database to a successful outbreak response. Such a database can be utilized to obtain information on the origin of strains, help relate sporadic cases, and improve estimates of the disease burden in order to increase the overall understanding of viral epidemiology. Foodborne viruses are not currently monitored by the Foodborne Diseases Active Surveillance Network (FoodNet) as they are not routinely tested for in clinical laboratories. Calicinet, the national norovirus outbreak surveillance system in the United States, has provided significant information on the genotypes of HuNoV preferentially associated with foodborne outbreaks, as well as the continuous tracking of newly emerging strains. Since both HuNoV and HAV may occasionally have more than one circulating strain simultaneously, it is important to identify the sequence of appropriate length and genome sections for surveillance purposes. For example, in a recent HAV outbreak, RNA from serum of 248 of 421 (59 %) reported case-patients could be sequenced. Without

this typing, foodborne transmission was suspected for only 4 % of reported case-patients. However, when sequence typing was applied, foodborne transmission increased to being the most probable source of infection for 16 % of the patients (Petrignani et al. 2014).

For HAV, sub-genomic region, such as the VP1/P2B junction of the capsid, are frequently used for sequencing. Since the HAV genome is highly conserved, strains that are not highly similar share common sequences in these sub-genomic regions. When different sub-genomic regions were recently evaluated, not one was identified that could accurately represent the phylogenetic relationship as accurately as when using whole genome sequences (Vaughan et al. 2014). As such, we recommend using whole genome sequences for more accurate source tracking, especially in linking dispersed clusters. Such an approach may be more profound in the case of HuNoV that are much more genetically diverse. Indeed, whole genome sequencing was used in an HuNoV outbreak in a clinical setting; the phylogenetic information was able to link and identify the direction of transmission enabling effective control measures to be put into place (Kundu et al. 2013). It has been similarly suggested that if typing of the complete HuNoV genome was included in future outbreak investigations, it could provide better outbreak resolution and help monitor emerging variants, which is of utmost value at coordinating international tracing efforts (Vega et al. 2014).

In conclusion, more efficient detection, prevention, and control measures for produce-associated virus outbreaks would require improvements in detection methodologies combined with enhanced epidemiological and microbiological surveillance data that are collected, correctly interpreted and shared internationally in an effort to promote risk communication and tackle unresolved cases.

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CHAPTER 7

Shellfish-Associated Enteric Virus Illness: Virus Localization, Disease Outbreaks and Prevention

Gary P. Richards

1. INTRODUCTION

Enteric viruses are responsible for the majority of foodborne illnesses. These viruses include caliciviruses (classified as noroviruses and sapoviruses); picornaviruses (hepatitis A virus [HAV] and aichivirus); hepatitis E virus (HEV); astroviruses; rotaviruses; enteric adenoviruses; coronaviruses; toroviruses; and picobirnaviruses. The most frequently reported foodborne outbreaks are caused by noroviruses; formerly called the agent of winter vomiting disease, Norwalk or Norwalk-like viruses, or small round structured viruses. Hepatitis A virus is also reported as a cause of foodborne illness albeit less frequently. Children are infected in early childhood with group A rotaviruses, enteric adenoviruses, astroviruses, and caliciviruses and may develop partial immunity against them (Glass et al. 2001). Molluscan shellfish are common vehicles for virus transmission and methods are available for the detection of a wide range of viruses in shellfish as well as in the stools of infected individuals (Le Guyader et al. 2008; Iizuka et al. 2010; Richards et al. 2015; Polo et al. 2015).

Enteric viruses have undoubtedly been infecting humankind since the dawn of civilization; however, techniques to isolate and identify these viruses are still under development. With the advent of sensitive molecular methods, even non-propagable viruses may now be detected. In spite of these advances, reporting of enteric viral illnesses is poor or non-existent in many parts of the world today. Noroviruses are believed to constitute the most frequent cause of foodborne illness; however, only major outbreaks are usually recorded and accurate, quantitative assessment of the number of individuals affected is often not available. Accountability for hepatitis A and hepatitis E infections is important due to the potential seriousness of the diseases. Symptoms of illness caused by enteric viruses vary, depending on the virus and the sensitivity of the infected individuals. In healthy individuals, hepatitis A is often an asymptomatic infection with spontaneous remission, thus the true incidence of hepatitis A infection remains uncertain. The incubation period for hepatitis A is generally 15–45 days and symptoms include nausea, vomiting, anorexia, malaise, fever, jaundice, and abdominal pain usually in the upper right quadrant

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(reviewed in Richards 2005). Liver damage can result from an HAV infection. Virus may be shed from infected individuals for up to 5 months (Rosenblum et al. 1991; Robertson et al. 2000). Similarly, hepatitis E can be a serious illness, but is so rare in the United States, that only a handful of cases have been recorded. In Asia and other parts of the world, outbreaks of hepatitis E are frequently encountered. The incubation period for hepatitis E is reportedly from 2 to 8 weeks and early symptoms may include vomiting, malaise, fatigue, anorexia, and low-grade fever ultimately leading to possible spleen enlargement and pain in the upper right quadrant, again from liver involvement (reviewed in Richards 2005). Clinical symptoms generally resolve within 4–8 weeks, except in pregnant women who have a 15–25 % mortality rate (Mast and Krawczynski 1996).

Symptoms of human norovirus (NoV) and sapovirus include vomiting, diarrhea, nausea, abdominal cramps, chills, fever, headache, body ache, and can lead to dehydration (reviewed in Richards 2005). The incubation period for NoV illness is 1–2 days after consumption of contaminated food or water. Symptoms usually clear spontaneously after about 2 days. NoV is believed to be the most prevalent cause of foodborne illnesses in the world today. When illnesses are noted, there is seldom epidemiological follow-up to confirm the cause. Most of the illnesses are likely from drinking sewage-contaminated water or the consumption of raw or undercooked foods that are tainted by contaminated water, the hands of food-handlers, or the transfer of viruses from contaminated contact surfaces to the food. The more serious viral illnesses are from HAV and HEV, which can lead to life threatening liver disease and even death. Death is seldom a consequence of NoV and related enteric viruses, although in rare cases, death may result from severe dehydration, particularly in regions of the world where rehydration therapy is not readily available.

Other viruses of interest from a shellfish safety standpoint include rotavirus and astrovirus (reviewed in Richards 2005). Rotaviruses generally produce diarrhea, anorexia, dehydration, occasional vomiting, and dehydration, and are most commonly observed in young children who have not yet developed immunity. Most shellfish consumers would be expected to be immune to rotaviruses from normal childhood exposure. Astroviruses can also be transmitted by shellfish and often produce a mild and self-limiting illness. Symptoms include occasional vomiting, diarrhea, fever, abdominal pain and anorexia. It has a short incubation period (1–3 days) and the illness lasts for up to 4 days. The incidence of illnesses from astrovirus, NoV, sapovirus, rotavirus, and related viral pathogens is underreported partly because these viruses cause illnesses of short duration, and seldom cause mortality or serious long-term illness or disability.

Among the most notable foods that may contain enteric viruses are raw or undercooked molluscan shellfish (oysters, clams, mussels, and cockles). Shellfish accumulate contaminants, including enteric viruses, from their surrounding waters and bioconcentrate them within their edible tissues.

Consequently, some very large outbreaks of HAV and NoV have been reported following consumption of contaminated shellfish. Efforts to document such outbreaks have provided some light on the causes and effects of shellfish-borne disease, but do not convey the magnitude of the problem (Gerba and Goyal 1978; Richards 1985, 1987; Rippey 1994). The latest estimates from the Centers for Disease Control and Prevention (CDC) indicate that NoVs are the most common cause of acute foodborne gastroenteritis in the United States and are responsible for an estimated 5.5 million cases annually (Scallan et al. 2011; Hall et al. 2012). The vast majority of illnesses go undiagnosed and, until recently, outbreak statistics have not been systematically maintained. Improvements in monitoring, such as the development of CaliciNet in the United States in 2009, are contributing to better reporting of NoV outbreaks including the specific genogroup (genogroup I or II) and genotype responsible for the illness (Vega et al. 2011). A National Outbreak Reporting System (NORS) was also established in 2009 to collect information on NoV and other waterborne and foodborne outbreaks according to mode of transmission, etiology and setting (Manikonda et al. 2012). In 2007, a market survey of oysters in the United States was conducted by the U.S. FDA. They detected NoV in 3.9 % of the oysters tested and HAV in 4.4 % of the oysters (DePaola et al. 2010). Market oysters were also tested in France with NoV detected in 9 % of the samples (Schaeffer et al. 2013). In stark contrast, a 2-year study of oysters from 39 commercial harvesting sites in the United Kingdom showed NoV in 76.2 % (643 out of 844) of the oysters and all sites tested positive for NoV at least once (Lowther et al. 2012b). This would suggest high levels of seawater contamination in the UK. It could not be determined from the above tests what portions of the viruses in the oysters were infectious and what portions had been inactivated.

Although persons infected with NoV can develop acute vomiting and diarrhea, symptoms are usually fleeting, lasting only a day or two. Consequently, the patients do not seek medical attention because symptoms resolve rapidly and spontaneously. Those who are ill may spread the disease to family members through contamination of surfaces or by handling foods with inadequately sanitized hands. A secondary attack rate among household contacts was reported as 14 % in one study (Alfano-Sobsey et al. 2012). Sick individuals often miss work for 2 or 3 days, but when they return, they may still carry the virus and be a source of infection to their workmates (White et al. 1986; Iversen et al. 1987; Haruki et al. 1991; Graham et al. 1994). A study by the CDC indicates that an estimated 5.5 million cases of foodborne NoV occur in the United States each year with 15,000 hospitalizations and 150 deaths (Scallan et al. 2011). Health care and lost productivity costs due to NoV in the United States are estimated at $2 billion annually (Batz et al. 2011).

The scientific literature contains numerous reports of disease outbreaks due to HAV and NoV in shellfish. Epidemiological linkage of an outbreak to a particular source is more difficult for some virus infections due to differences in incubation times. For instance, HAV has an incubation period of approxi-

mately 1 month and sick individuals may not be able to say with any degree of certainty where they ate or what they ate a month earlier. However, larger outbreaks are more likely to reveal the source of infection, whether it is water, food, or from a party or restaurant. Sources of NoV and sapovirus illnesses are easier to track because of the viruses' relatively short (1–2 day) incubation period. Rotavirus causes diarrhea in infants and young children and, although it may be transmitted by foods, children often develop immunity to rotavirus at an early age. Rotavirus diarrhea may lead to dehydration and vascular collapse, particularly when rehydration therapy is not available. Although rotavirus is transmitted by the fecal-oral route, it is likely that most illnesses are from direct contact with children and fomites, rather than through the foodborne route. Astrovirus is another pathogen that has been difficult to track. Molecular diagnostic methods are now available for astroviruses, which may allow more screening of foods for the virus, especially in outbreak investigations.

2. VIRUS LOCALIZATION WITHIN SHELLFISH

Molluscan shellfish feed by filtering materials out of their surrounding water, a process referred to as bioconcentration. In this process, bivalve shellfish collect contaminants to levels much higher than in their surrounding environment. Estimates are that shellfish can bioconcentrate enteric viruses to 100 times the level in seawater. Materials filtered out of the water constitute food and include viruses, bacteria, algae, and other materials. Bioconcentation is accomplished by the initial filtration of viruses, often adsorbed to particulates, from the water by the gills. From the gills, food is diverted to the mouth where it travels to the digestive tract, which includes the stomach and digestive diverticula. A portion of the food in the digestive tract passes through the shellfish, ending up as feces, but some of the food is taken up by motile phagocytic hemocytes, which pass from the blood stream of the shellfish into and out of the lumen of the gut. It is these motile phagocytic hemocytes that are essential to carrying nutrients required to support the nutritional needs of the shellfish tissues. Viruses in environmental waters are often adsorbed to particulates, making them large enough to be filtered out by bivalves. Once within the digestive tract, viruses may be phagocytized by hemocytes where they are carried to tissues surrounding the gut, including epithelial tissues of the stomach and more distant connective tissues (Le Guyader et al. 2006b; Seamer 2007; McLeod et al. 2009; Richards et al. 2010). Hemocytes are known to contain acidic vacuoles and various digestive enzymes to degrade the complex foods into forms more readily absorbed by the cells of the mollusk. A recent study showed that the duration of virus persistence within the

hemocytes depends on the acid stability of the virus, with HAV, murine norovirus, poliovirus, and feline calicivirus persisting for 21 days, 12 days, 1 day, and <1 day, respectively (Provost et al. 2011).

Studies on enteric virus retention by shellfish have shown that viruses can persist for periods significantly longer than bacterial indicator organisms, like *Escherichia coli* and fecal coliforms (Cook and Ellender 1986; Power and Collins 1989, 1990). Once within the tissues surrounding the gut, virus elimination by simple passage through the intestines via the feces is no longer an option. Early work on virus localization in the Pacific oyster (*Crassostrea gigas*) was performed in New Zealand using cricket paralysis virus (an insect picornavirus) (Hay and Scotti 1986); the virus was found not only in the lumen of the stomach but also in the stomach epithelium and digestive diverticula following a period of virus uptake. Similar results were observed with human pathogenic viruses. For instance, NoVs were detected in the lumen of the gut of Pacific oysters after bioaccumulation of virus-contaminated seawater (Le Guyader et al. 2006b). Norovirus was also detected in the lumen of the stomach and in epithelial tissues surrounding the stomach and digestive diverticula of Pacific oysters after viruses were bioconcentrated (McLeod et al. 2009; Seamer 2007; Richards et al. 2010). Another study showed the localization of HAV in basal cells of the ciliated epithelium of the stomach and in the hepatopancreas of Eastern oysters (*Crassostrea virginica*) (Romalde et al. 1994). Poliovirus was also shown to have a similar fate upon bioconcentration in oysters (Richards et al. 2010). Thus, the persistence of viruses in contaminated shellfish appears to be related to the sequestration of viruses from the lumen of the gut to tissues surrounding the gut and to hemocytes which may retain viable virus for extended periods.

Shellfish depuration is a commercial process which is widely used worldwide to purge microbes and other contaminants from shellfish (reviewed by Richards 1988). It involves the purging of microbes and other materials from bivalve shellfish by placing them in tanks of clean seawater, often recirculated and disinfected by means of ultraviolet light, ozone, or other means. Depuration is practiced widely throughout Europe, New Zealand and parts of Australia. In the United States, "approved" shellfish growing waters are widespread; thus, depuration is only occasionally practiced. The depuration process is usually performed for about 3 days, although in some places, like New South Wales, Australia, only 36 h of depuration are required. The overall success of the depuration process is determined by reductions in bacterial counts, often using fecal coliform bacteria as indicators. The translocation of viruses from the lumen of the digestive tract to tissues surrounding the tract, and the overall resilience of viruses to the effects of various digestive processes within the hemocytes renders depuration of shellfish relatively ineffective from the context of virus removal.

3. CASE STUDIES

Since reporting of viral illnesses and their association with a particular food are inadequate at best due to poor reporting practices, this section will not attempt to tabulate and list outbreaks by country or food source. Instead, the focus will be on highlighting specific, shellfish-related outbreaks by known shellfish-borne viral pathogens in countries around the globe and to indicate sources of contamination, when known.

3.1. Hepatitis A Virus

The United States has experienced numerous outbreaks of hepatitis A associated with shellfish. Major reported outbreaks date back to 1961 with 459 cases in New Jersey and New York from the consumption of clams; 372 cases in Pennsylvania, Connecticut, and Rhode Island in 1964 from clams; and 293 cases in Georgia, Missouri, New Mexico, Oklahoma, and Texas in 1973 from oysters from Louisiana (reviewed in Richards 1985). Oysters associated with the 1973 outbreaks were consumed raw, but were reportedly obtained from waters that met the bacterial standards of the National Shellfish Sanitation Program (Portnoy et al. 1975; Mackowiak et al. 1976). Flooding of polluted Mississippi River water into oyster growing areas occurred 2 months earlier and may have been responsible for the outbreaks (Portnoy et al. 1975; Mackowiak et al. 1976). A multistate outbreak of hepatitis A was attributed to the consumption of raw oysters from Florida (Desenclos et al. 1991). The attack rate was calculated at 19 persons per 10,000 dozen oysters consumed in restaurants.

The largest outbreak of hepatitis A on record occurred in and around Shanghai, China, from January through March, 1988. Over 293,000 individuals became ill after eating clams harvested from recently opened mud flats outside of Shanghai (Xu et al. 1992) with 47 deaths reported (Cooksley 2000). Most of the cases were associated with direct consumption of the clams, rather than from person-to-person transmission. Since the incubation period to develop hepatitis A is around 30 days, many people had eaten the clams before any illnesses were apparent. During this same period, factory workers in Shanghai also developed hepatitis A after eating raw and cooked clams (Wang et al. 1990; Halliday et al. 1991; Tang et al. 1991). Since thorough cooking is known to inactivate enteric viruses, it appears that the clams were not fully cooked or were re-contaminated after cooking. Between 1976 and 1985, there were 109 cases of hepatitis A reported in Japan and 11 % were believed to be from consuming raw shellfish (Kiyosawa et al. 1987; Konno et al. 1983). Another study reported 225 cases of hepatitis A in Japan with raw oysters being the likely vehicle for infection (Fujiyama et al. 1985).

In 1997, 467 cases of hepatitis A occurred in New South Wales, Australia, from the consumption of oysters harvested from Wallis Lake (Conaty et al. 2000). One person died and a class action suit was filed on behalf of the victim and those who became ill. Before marketing, the government of New South Wales requires that all shellfish be subjected to the commercial process of depuration.

Depuration has been shown to be effective in eliminating many bacterial pathogens and spoilage organisms from molluscan shellfish, but does not completely eliminate enteric viruses such as HAV and NoVs (Richards 1988; Richards et al. 2010). Long-term relaying may be a better alternative to naturally purging viruses from shellfish. Relaying is when shellfish are removed from marginally polluted growing areas and replanted into clean waters for an extended period, often ≥10 days, to more extensively purge contaminants (reviewed in Richards 1988). This duration provides safer shellfish but some viruses (e.g., HAV) have been shown to persist in a viable state for up to 3 weeks in oysters (Kingsley and Richards 2003).

Europe too has had its share of hepatitis A outbreaks associated with contaminated shellfish. Outbreaks of hepatitis A associated with the consumption of oysters, cockles, and mussels have been reported in England, Wales, and Ireland (Maguire et al. 1992; O'Mahony et al. 1983; and Polakoff 1990). An outbreak of hepatitis A from imported clams, with secondary spread to a public school, was reported in Italy (Leoni et al. 1998). The total cost of one outbreak of hepatitis A involving 5889 cases in Italy was estimated at $24 million while costs to each sick individual were estimated at $662 (Lucioni et al. 1998). Raw mussels and clams were the apparent vehicles of transmission for an outbreak of HAV in Italy and a dose-response relationship was observed between illness and the amount of shellfish consumed (Mele et al. 1989). Spain experienced HAV outbreaks in 1999 with 184 cases from the consumption of clams meeting European Union standards (Sanchez et al. 2002). Clams imported from Peru led to 183 cases of hepatitis A in Spain and the virus was detected in 75 % of the clam samples tested (Bosch et al. 2001). A survey of South American imports showed the presence of HAV in 4 of 17 lots of mollusks (Romalde et al. 2001). The outbreak of hepatitis A associated with imported frozen clams led to a call for improved risk assessment to prevent such outbreaks (Pintó et al. 2009).

3.2. Noroviruses

A review of the early literature indicates 6049 documented cases of shellfish-associated gastroenteritis in the United States between 1934 and 1984 (reviewed by Richards 1987). Since no bacterial pathogens were associated with these illnesses and symptomology was consistent with NoV illness, it seems likely that NoVs were the causative agents. One outbreak involved 472 cases of gastroenteritis from the consumption of Louisiana oysters. This outbreak resulted in 25 % of Louisiana's one-quarter million acres of shellfish beds being closed, an estimated loss to the industry of $5.5 million, and disruption of harvesting for 500 licensed oystermen (Richards 1985). Some outbreaks were small, such as the one in Florida in 1980 involving only six individuals who ate raw oysters (Gunn et al. 1982). In another case, oysters from a defined area in Louisiana were associated with outbreaks of NoV illness in at least five states: Louisiana, Maryland, Mississippi, North Carolina,

and Florida (Centers for Disease Control 1993). Although these oysters were distributed throughout the United States, outbreaks were identified only in these five states. Identification of the source of contaminated shellfish was facilitated by tags (labels) that had been placed on sacks of oysters indicating, among other things, the location of harvest. Shellfish tagging is commonly required by regulators in order to facilitate shellfish tracking in the event of an outbreak.

The worst period on record for NoV outbreaks in the United States was in 1982–1983 when New York experienced numerous outbreaks associated with raw and steamed clams (Centers for Disease Control 1982; New York State Department of Health 1983) and from oysters (Morse et al. 1986). At least 441 people developed acute gastroenteritis and eight of these individuals subsequently developed hepatitis A as well. Ten outbreaks during the summer were attributed to the illegal harvesting of oysters by an unlicensed digger in polluted waters that were closed to shellfishing along the Massachusetts coast (Morse et al. 1986). Other contaminated shellfish were obtained from Rhode Island waters. Another series of outbreaks in the winter was from clams harvested in New York waters. Negative publicity and the lack of confidence in the safety of local shellfish prompted shellfish dealers to obtain clams depurated in England. Unfortunately, these clams led to over 2,000 illnesses in 14 separate outbreaks in New York and New Jersey over a 3-month period (Richards 1985). These clams, served at a picnic, were responsible for over 1100 cases of NoV illness in one outbreak. The U.S. Food and Drug Administration investigated the outbreaks and concluded that depuration was poorly monitored in plants from which the shellfish were obtained (Food and Drug Administration 1983). Indeed, depuration itself may contaminate shellfish if the waters used for depuration are compromised, as may have occurred in an outbreak of hepatitis A involving 111 individuals in France (Guillois-Bécel et al. 2009).

An outbreak of NoV gastroenteritis occurred in 1983 in Rochester, New York. A survey indicated that 84 (43 %) of 196 people interviewed had NoV-like symptoms after eating "cooked" clams served at a clambake. The clams were harvested off the coast of Massachusetts from waters known to be contaminated by untreated municipal sewage (Truman et al. 1987). This outbreak may have been avoided if the clams had been fully cooked or if the shellfish had been obtained from waters meeting the standards of the National Shellfish Sanitation Program. Several other NoV outbreaks in the United States have been associated with cooked oysters (Kirkland et al. 1996; McDonnell et al. 1997). In an outbreak of NoV gastroenteritis that affected 129 individuals in Florida in 1995, sick individuals had eaten raw, cooked, and what were reported to be thoroughly cooked oysters (McDonnell et al. 1997). Those who ate the so called thoroughly cooked oysters made a subjective judgment on the degree to which their shellfish had been cooked; it is unlikely that thoroughly cooked oysters would cause illness unless they were re-contaminated after cooking, perhaps by dirty gloves used during shucking, by use of contaminated shucking knives, or by placing cooked product on unsani-

tized tables or on NoV-contaminated ice. There was speculation that the source of the NoV contamination was the overboard dumping of sewage in the oyster harvesting area (McDonnell et al. 1997). This is not the first instance when overboard disposal of feces or vomit led to contaminated shellfish beds followed by outbreaks of illness. Kohn et al. (1995) conducted a survey of crew members from oyster harvesting boats and learned that 85 % of the boats disposed of sewage overboard. Although this is against regulations, monitoring for compliance is very difficult. Berg et al. (2000) also reported the overboard disposal of sewage by oyster harvesters in Louisiana as the likely source of contaminated oysters in at least two outbreaks. New Zealand experienced a number of oyster-associated outbreaks of NoV illness and overboard disposal of sewage from recreational boats was suggested as a likely source of contamination (Simons et al. 2001). Likewise, an outbreak of oyster-associated NoV gastroenteritis in Canada was suspected to be from contamination by an ill harvester (McIntyre et al. 2012).

A study by the CDC (Hall et al. 2012) gave a breakdown of foodborne NoV outbreaks in the United States from 2001 to 2008; a total of 2,922 outbreaks were reported of which 13 % were attributable to shellfish, 16 % to fruits and nuts, and 33 % to leafy vegetables (Hall et al. 2012). A comprehensive review of world literature from 1980 to 2012 showed that shellfish were responsible for an estimated 359 viral outbreaks of which 83.7 and 12.8 % were ascribed to NoV and HAV, respectively, with oysters causing an estimated 58.4 % of the illnesses (Bellou et al. 2013).

Other countries have also experienced shellfish-associated NoV outbreaks. For example, a widespread outbreak of NoV illness involving over 2,000 people occurred in Australia in 1978 and was subsequently linked to oyster consumption (Murphy et al. 1979; Grohmann et al. 1980). Another outbreak in Australia affected 25 of 28 people who ate raw oysters at a hotel (Linco and Grohmann 1980). In response to these outbreaks, in 1981, the government of New South Wales, Australia, implemented regulation requiring that all shellfish be subjected to depuration (Ayers 1991). A study was commissioned to determine whether depurated oysters from two sites in Australia would cause illness in human volunteers (Grohmann et al. 1981). Depurated oysters from one site produced NoV illness in 52 people but those from the second site did not. Oysters were also the presumptive vehicle of NoV transmission to residents of New South Wales and Queensland in a 1996 outbreak involving 97 cases (Stafford et al. 1997). More recently, an outbreak involving 306 cases of NoV illness were reported from oysters in Tasmania (Lodo et al. 2014).

In Japan, both oysters and clams have been associated with NoV outbreaks. A study of 80 outbreaks of acute gastroenteritis from 1984 to 1987 revealed that 53 were associated with the consumption of oysters (Sekine et al. 1989). Another study reported five outbreaks of NoV illness from eating raw oysters (Otsu 1999). In a review of NoV outbreaks in Okayama, Japan, over a 5 month period, 9 of 46 outbreaks (20 %) were attributed to shellfish (Hamano et al. 2005). A study involving 286 fecal specimens from 88 oyster-associated out-

breaks of illness in Japan showed that NoV was associated with 85 out of 88 (96.6 %) of the outbreaks, and 197 of 286 (68.9 %) of the fecal specimens analyzed were positive for NoV (Iritani et al. 2014). Clams imported from China caused 22 cases of NoV gastroenteritis and four cases of hepatitis A in Japan (Furuta et al. 2003). Chinese clams imported into the United States and served in a restaurant were associated with five cases of NoV illness in New York (Kingsley et al. 2002b). Regulations required that the clams be cooked before import to the United States. Although these clams were labeled as cooked, they had the appearance of raw product. Molecular analyses detected both NoV and HAV in the clams, although no hepatitis A cases were reported. Extremely high levels of fecal coliforms were also detected in the clams (Kingsley et al. 2002b).

NoV outbreaks in Europe have also been reported. Cockles were linked to an early outbreak of NoV (Appleton and Pereira 1977). Mussels were responsible for an outbreak at a national convention in the United Kingdom and a dose response relationship was noted (Gray and Evans 1993). English oysters that had been depurated and served at a birthday party caused nine cases of NoV gastroenteritis (Ang 1998). Oysters from France were associated with NoV illness in 127 French and 200 Italian consumers (Le Guyader et al. 2006a). Contamination of the oysters was associated with a heavy rain event. Lowther et al. (2012a) reported a dose-response relationship between NoV contamination level and infection risk. Oysters implicated in an outbreak contained significantly higher levels of NoV RNA than oysters taken from commercial production areas where outbreaks were not observed.

3.3. Hepatitis E Virus

Like other enteric viruses, infection with HEV occurs via the fecal-oral route. It is a major cause of epidemic as well as sporadic viral hepatitis in endemic regions of Asia, the Indian subcontinent, Africa, and the Americas (Arankalle et al. 1994; Balayan 1997; Velazquez et al. 1990; Clayson et al. 1997). Hepatitis E is less frequently detected in Europe and only a handful of cases have been reported in the United States. In some developing countries, HEV may account for over 50 % of acute viral hepatitis cases (Balayan 1997; Clayson et al. 1997). Like HAV, HEV normally causes an acute, self-limiting disease with a low mortality rate; however, during pregnancy mortality rates between 15 and 25 % have been reported (Mast and Krawczynski 1996). Epidemiological studies have shown that transmission of HEV occurs predominately by ingestion of contaminated water (Arankalle et al. 1994; Balayan 1997), with low incidence of person-to-person or foodborne transmission established to date. Shellfish consumption was considered a risk factor for sporadic cases of hepatitis E in Eastern Sicily (Cacopardo et al. 1997) and undercooked cockles and muscles were associated with HEV infection in India (Tomar 1998). Epidemiological follow-up is difficult with this virus because of a 15–60 day incubation period and the sporadic distribution of illnesses. To date, no large outbreaks of shellfish-associated hepatitis E have been reported although it

should be considered a potential emerging pathogen which may in time pose a more serious threat in the United States and other countries. A study of 286 shellfish samples collected from environmental sites impacted by pigs, wild boars and human waste in France failed to show the presence of HEV (Grodski et al. 2014), even though HEV is known to be zoonotically associated with pigs. In Scotland, 36 of 39 mussels (92 %) from the West Coast and 5 of 9 (55 %) mussels from the East Coast were reportedly positive for HEV, primarily genotype 3, at levels between 3.7 and 5.2 $\log_{10}$ RT-PCR units per ml (Crossan et al. 2012). Genotype 3 HEV was also detected in 2 out of 32 packages (1.6 %) of freshwater bivalves (*Corbicula japonica*) obtained from a fish market in Japan (Li et al. 2007). The source of the contamination was speculated to be from wildlife, possibly deer and wild boar.

4. DISEASE PREVENTION

4.1. Routine Monitoring and Regulations

The United States and the European Union (EU) have implemented criteria for the harvesting and processing of molluscan shellfish. Under the guidelines of the National Shellfish Sanitation Program (Anon. 2011), shellfish harvesting in the United States has been historically based on water quality criteria derived from sanitary surveys of shellfish growing water. The surveys are based on the levels of total or fecal coliforms in the water and are determined during periodic water sampling and testing. Water testing has served the country well since its implementation in 1925 (Frost 1925). Sanitary surveys were originally undertaken to reduce the incidence of typhoid fever among shellfish consumers and a successful outcome was achieved. Today, shellfish growing waters are classified as approved, conditionally approved, restricted, conditionally restricted, or prohibited, depending on the level of coliform contamination.

According to the National Shellfish Sanitation Program guide (Anon. 2011), shellfish obtained from waters with a most probable number (MPN) of fecal coliforms <14/100 ml are classified as approved for shellfish harvesting and direct sale. Shellfish waters are classified as restricted if the fecal coliform levels are under 88/100 ml, while shellfish are prohibited from harvest when the waters have an MPN >88 fecal coliforms/100 ml. Since water classification is an ongoing process and the history of a site can be determined by an examination of past data, some areas with intermittent contamination may be classified as conditionally approved and conditionally restricted. Such waters come under a management plan and shellfish are permitted to be harvested for direct sale or for depuration/relaying when the criteria of the plan are met. Shellfish from restricted areas can be harvested only if they are subjected to depuration or relaying before they enter the marketplace. Shellfish from prohibited areas may never be harvested or marketed.

Table 7.1 Council directives for the production and marketing of shellfish according to European Union standards based on fecal coliform or *E. coli* levels in the meats (Anon. 1991)

Classification	*Fecal coliform limit*	*E. coli limit*
A—sell without processing	<300 MPN/100 g	<230 MPN/100 g
B—depurate or relay	<6,000 MPN/100 g	<4,600 MPN/100 g
C—prolonged relay	<60,000 MPN/100 g	NA
D—prohibited	>60,000 MPN/100 g	NA

NA: not applicable

In contrast, the EU follows Council Directive 91/492/EEC (Anon. 1991), which regulates shellfish based on the levels of fecal coliforms or *E. coli* in the shellfish meats, rather than in the shellfish growing waters. Under this system, shellfish meats are classified in one of four categories: A, B, C, or D, as shown in Table 7.1. The number of fecal coliforms and *E. coli* are determined by MPN and the results are reported per 100 g of shellfish meat. The differences between the US standard, which is based on water quality criteria, and the EU standard, which is based directly on shellfish quality criteria, have led to some disagreement between government regulators of the two regions. However, both standards contribute significantly to the reduction of shellfish-borne illness from bacterial contaminants although their effectiveness in reducing viral illnesses remains unknown. There are many shellfish growing waters in the US that are perceived to be clean enough for direct harvest and sale of shellfish, whereas, shellfish meats are seldom clean enough in the EU for direct shellfish harvest and sale. As a consequence, most shellfish in the EU must be depurated or relayed before they are marketed. In contrast, depuration is seldom required in the US. Regardless of which standard is used, the levels of fecal coliforms are not a good indicator of the virological quality of shellfish, because enteric viruses persist longer than coliforms within shellfish tissues and they depurate poorly. Therefore, reliance on coliforms as a predictive index for virus presence is not very effective. Only when coliform levels are high do the standards prevent the direct sale of potentially virus-laden shellfish. Unfortunately, viruses tend to be more resilient to the effects of sewage treatment processes and environmental stressors than coliforms and hence water containing low or negligible levels of indicator bacteria may still contain high levels of enteric viruses.

Shellfish growing waters are often impacted by the disposal of sewage from commercial and recreational vessels (Kohn et al. 1995; McDonnell et al. 1997; Simons et al. 2001) leading to sporadic contamination events that are difficult to assess by either the US or EU methods. In a Florida outbreak, the attack rate for HAV in seafood establishments was estimated to be 1.9 per 1,000 dozen oysters eaten (Desenclos et al. 1991). Such low-level contamination would likely miss detection using the EU meat standard, because of the low

number of samples tested, the likely randomness of contamination, and the lack of correlation between coliforms and enteric viruses within the meats. In contrast, the utility of the water standard is also limited by the lack of correlation between coliforms and viruses, the generally lower numbers of coliforms (and viruses) in the water compared to the meats, and the lack of homogeneity of the water due to tides, winds, currents, and point source contamination events. One benefit of the water standard is that over many years of monitoring, the history of a particular water body becomes known such that predictions may be made for areas that are likely to be hot spots for fecal contamination versus those that are more likely to be less problematic.

4.2. Enhanced Monitoring and Enforcement

Monitoring of shellfish and their harvesting areas coupled with enforcement of regulations are both essential to shellfish safety. A number of areas are in need of better monitoring and enforcement if outbreaks are to be curtailed. Tighter enforcement of laws against dumping waste in shellfish harvesting areas would reduce the incidence of enteric virus illness. An area in need of enhanced monitoring is the illegal practice of harvesting shellfish from closed areas, a practice called poaching or bootlegging. Some outbreaks have been attributed to the sale and consumption of poached or bootlegged shellfish (Morse et al. 1986; Desenclos et al. 1991). Typically, the penalties for those who perpetrate such crimes have been relatively small. According to US and EU guidelines, all lots of shellfish must contain tags (US) or health marks (EU), which label the lot with information that allows the shellfish to be tracked to their source. This is important in outbreak investigations as health authorities seek epidemiological evidence to curb the spread of disease. Enhanced monitoring of tags and health marks would serve as a deterrent against poachers.

Tighter enforcement of import laws are needed to restrict the importation of tainted shellfish. Shellfish exported from China, England, Ireland, Peru, and many other countries have been apparent vehicles of enteric virus illness. Exporting countries are required to subscribe to the standards in place for the receiving country. Transactions are often sealed with a memorandum of understanding (MOU) between the exporting and importing nations. Failure to comply with the MOU would impose dire consequences upon the exporting country, including the withdrawal of the MOU in cases which show wanton disregard for the requirements of the agreement. Harvesters, processors, and shippers should meet criteria deemed necessary to ensure the safety of their merchandise. Hazard analysis critical control point (HACCP) plans should be in place to monitor factors that are important in ensuring shellfish safety. Practices to restrict the presence of fecal pollution in shellfish will likely reduce the incidence of bacterial and enteric viral illness in shellfish consumers, although direct measure of the benefits in regard to possible viral loads is difficult to ascertain.

4.3. Improved Sewage Treatment

Another intervention to reduce virus levels in shellfish would be to improve upon sewage treatment plants and septic systems, particularly in coastal regions near rivers, lakes, and shellfish-growing areas. Adequate monitoring and maintenance of treatment facilities are important to reduce viral loads emitted into the environment. The US routinely chlorinates effluent wastewater and this practice has some penetrating effects on particulate matter that contains potential pathogens. After treatment, the chlorine may be inactivated with sodium thiosulfate. In contrast, the EU often uses ultraviolet irradiation to treat sewage effluent. The lack of penetrating ability, particularly in turbid water or in water containing particulate matter, and the lack of any residual properties imparted by the UV would be expected to allow some viruses and bacteria to escape inactivation. The technology is available to eliminate or substantially reduce enteric viruses from sewage; however, few if any engineers have designed sewage treatment facilities with virus reduction in mind. Treatment plant maintenance and operation should be tightly controlled so that the facility works at its optimal efficiency. Controls should be in place to prevent or reduce accidental releases of untreated sewage during flooding events.

4.4. Analytical Techniques

Monitoring for viruses in water or shellfish is encouraged using molecular methods, namely reverse transcription-polymerase chain reaction (RT-PCR). New RT-PCR protocols continue to be developed along with improved methods to extract the viruses from water and shellfish (reviewed in Richards et al. 2015). Unfortunately, such methods are limited in their practical application because they fail to differentiate infectious from non-infectious viruses (Richards 1999). Direct assays for infectious viruses would be desirable; however, wild-type HAV, HEV, NoVs, sapoviruses and astroviruses have been either difficult or impossible to propagate or to assay in common laboratory animals. Recently, a method was described for the propagation of NoV in mice but it needs further validation (Taube et al. 2013). Even more recently, Jones et al. (2014) published a promising study reporting the successful propagation of NoV in a human lymphoblastoid B cell line. NoV replication occurred in the presence of enteric bacteria that express histo blood group antigens where viral genome copy numbers increased up to 25-fold by 5 days post infection. Viral structural and non-structural proteins also increased. They also reported a nearly 600-fold increase in viral genomes in a co-culture of human lymphoblastoma B cells and HT-29 intestinal epithelial cells after 3 days (Jones et al. 2014). Validation of their procedures is likely underway. Another technique which offers some promise for identifying infectious NoV is based on the binding of infectious NoVs to porcine mucin followed by quantitative RT-PCR (Dancho et al. 2012). NoV that was inactivated by heat, ultraviolet light or high pressure processing failed to bind to porcine mucin

(Dancho et al. 2012), suggesting that the porcine mucin binding assay coupled with quantitative RT-PCR may be a breakthrough in the search for a method to identify infectious NoVs in extracts obtained from shellfish and other foods. Many strains of wild-type HAV can be difficult to propagate in cell culture, and cell culture systems for HEV remain elusive. New virus propagation assays are needed to adequately assess shellfish safety from a virological perspective.

4.5. Processing Strategies

As mentioned in section 2.0, shellfish depuration is only minimally effective in reducing enteric viruses due to virus migration from the gut to tissues surrounding the gut. Long-term relay of shellfish from marginally contaminated waters to clean waters is more likely to render shellfish safer (Richards 1988); however, the exact duration required for relay is uncertain and is dependent on many factors including seawater temperature, shellfish species, level of initial contamination, virus type, etc. Intervention strategies to reduce or eliminate enteric virus contamination in shellfish should be implemented on multiple fronts and lessons from previous outbreaks should be heeded. Perhaps the simplest intervention available to consumers is cooking. In most outbreaks, raw or lightly cooked mollusks appear to be the primary vehicles of infection. Alternative processing strategies, like irradiation and high hydrostatic pressure processing, have been proposed. The high levels of irradiation required to inactivate enteric viruses imparts undesirable flavor characteristics to shellfish meats. On the other hand, high hydrostatic pressure processing for 5 min was shown effective in inactivating 7-$\log_{10}$ of HAV and feline calicivirus, a surrogate for NoVs (Kingsley et al. 2002a). High pressure inactivates viruses by denaturation of capsid proteins (Kingsley et al. 2002a) and sanitizes the shellfish from bacterial pathogens and spoilage organisms as well. Human NoV was successfully inactivated in oysters using high pressure processing, as determined through clinical trials using pressure-treated oysters (Leon et al. 2011).

4.6. Disease Reporting and Epidemiological Follow-Up

Improved reporting and epidemiological follow-up are needed to understand the magnitude of enteric virus outbreaks and to stop outbreaks once they occur. Such reporting has been effective in Italy where 35 participating local health units link incidence notification with serology and follow-up questionnaires in their surveillance for HAV (Mele et al. 1986, 1997). In a survey of ten EU countries, eight had national databases for hepatitis A statistics (Lopman et al. 2002). Likewise, the CDC has maintained statistics on reported cases of hepatitis A in the US. Although some countries maintain statistics on the number of cases of hepatitis A reported, few determine the source of the illness due to the high cost for epidemiological follow-up. NoV illness is not a notifiable disease in most countries, meaning that there are no formal, mandated systems requiring that illnesses be reported.

4.7. Hygienic Practices

Most outbreaks of shellfish-associated viral illness appear to be from shellfish contaminated within their natural environment. However, some cases, particularly those involving cooked shellfish, may actually be from product contamination by shuckers, handlers, or fomites. The contamination of foods by unsanitized hands of food handlers has led to numerous outbreaks of hepatitis A and NoV (Richards 2001). Better enforcement of hand washing practices may prevent some potential outbreaks from becoming a reality. Likewise, sanitary standards generally applied in the food industry should be enforced in the shellfish industry, especially on harvesting boats, in processing plants, transport facilities, and restaurants. Better education and monitoring of food handlers are needed to ensure compliance with food sanitation requirements.

5. SUMMARY

Numerous outbreaks of shellfish-borne enteric virus illness have been reported worldwide. Most notable among the outbreaks are those caused by NoV and HAV. Lessons learned from outbreak investigations indicate that most outbreaks are preventable. Anthropogenic sources of contamination will continue to invade shellfish growing waters. Shellfish, by their very nature, will continue to bioconcentrate these contaminants, including enteric viruses. There is no quick fix for enteric virus contamination of shellfish; however, vigilance on behalf of the industry, regulatory agencies, and the consumer could substantially reduce the incidence of illness. Enhanced monitoring in all areas of shellfish production, harvesting, distribution, and processing would help to reduce viral illnesses. Pollution abatement and improved hygienic practices on behalf of the industry and consumers are needed. Improved analytical techniques for the detection of enteric viruses in shellfish will lead to enhanced shellfish safety and better protection for the consumer and the industry. Better reporting and epidemiological follow-up of outbreaks are keys to reducing the transmission of foodborne viral infections. It is anticipated that recent advances in analytical techniques, particularly for NoV, will lead to better monitoring capabilities for food and water and a reduction in the incidence of enteric virus illness among shellfish consumers.

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CHAPTER 8

Outbreaks and Case Studies: Community and Food Handlers

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1. INTRODUCTION

The onset of symptoms due to foodborne viral illness may occur within hours or even days after consumption of virus-contaminated foods, making it difficult to determine the source of contamination. The difficulties associated with virus detection in contaminated foods are amplified by the fact that most common foodborne viruses cannot be easily propagated in vitro. Hence, we seek information on the prevalence and spread of foodborne viruses through epidemiological investigations that offer more specifics regarding virus dose and transmission. Testing of clinical samples obtained through these investigations offer additional information, which may aid in the determination of what foods might have been contaminated.

Virus transmission by food handlers is an important aspect of foodborne viral outbreaks. Recent increases in the number of outbreaks where food workers have been implicated may be due in part to increased trade of food and ease of travel (Todd et al. 2007). Various factors influencing this increase are complicated and still not well understood. Worker involvement in outbreaks of human norovirus (HuNoV) and other enteric viruses is understood to a point, but in many cases the details are not well documented. However, there are decades-old outbreaks in which the details were reported and today they serve as excellent learning tools (Grieg et al. 2007).

In a review of various outbreak reports several factors were identified as causing or leading to food contamination (Grieg et al. 2007) including contamination of raw products and ingredients through animals or the farm environment; cross-contamination from raw ingredients of animal origin; bare-hand or even gloved-hand contact by handler, worker, or preparer; food handling by an infected person or carrier of pathogen(s); inadequate cleaning of processing and/or preparation equipment or utensils; failure to properly wash hands when necessary; allowing foods to remain at room or warm outdoor temperature for several hours (e.g., during preparation or holding for service); slow cooling (e.g., deep containers for large roasts); inadequate cold-holding temperatures;

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preparing foods a half day or more before service; insufficient time and temperature during hot holding; insufficient time and temperature during initial cooking and heat processing; and insufficient thawing followed by insufficient cooking.

Human norovirus has been identified as the etiologic agent of a majority (60.2 %) of 816 outbreaks documented from the 1920s to 2006 in which food handlers were involved (Grieg et al. 2007). Methodology was not available to detect HuNoV in a majority of these outbreaks and hence, there is no information on the source of these outbreaks. In several of these outbreaks, rapid transmission was likely through close contact of individuals in fast food chains and cruise ships, which continues today.

In addition, secondary spread, which is the spread of disease to others from cases exposed though the original source of infection, is an important and under-recognized cause of gastrointestinal illnesses. At times these cases can be detected on an epidemiological curve. In one study, secondary spread was identified in 8.8 % of cases in 936 households with acute gastroenteritis. The cases were possibly caused by HuNoV but no definitive agent was identified (Perry et al. 2005). In another study, 15 % of cases in a large HuNoV outbreak at a school associated with a water fountain were due to secondary spread (Hoebe et al. 2004). Secondary spread is more likely associated with confined units (Grieg et al. 2007); for example, a HuNoV outbreak was documented in a hotel kitchen where infected workers spread the virus to other staff members remote from the kitchen by direct and indirect contact (Nelson et al. 1992). In outbreaks of hepatitis A virus (HAV), immunoglobulin may be administered to exposed persons, but HAV typically generates more secondary cases (as high as 45 %) than other agents. These secondary cases typically do not receive immunoglobulin (CDC 1990).

This chapter illustrates issues of viral contamination and transmission via foods using case studies to describe the potential risks associated with five pertinent foodborne viruses e.g., HuNoV, rotavirus, HAV, Aichi virus, and hepatitis E virus (HEV).

2. HUMAN NOROVIRUS

Human norovirus is a leading cause of acute viral gastroenteritis. The Centers for Disease Control and Prevention (CDC) estimates that HuNoV causes the most foodborne illnesses each year in the U.S. accounting for 58 % of all foodborne illnesses (Scallan et al. 2011). Recent studies revealed that HuNoV was also a leading cause of pediatric gastroenteritis in the U.S. associated with nearly 1 million health care visits annually. The total number of illnesses of HuNoV in the U.S. each year is 19–21 million, with an average of 5 episodes of HuNoV gastroenteritis experienced by each individual in his/her lifetime (Hall et al. 2013; Payne et al. 2013).

Noroviruses are classified into six genogroups I-VI (GI-GVI); the human strains belong to genetic clusters within GI, GII, and GIV (Zheng et al. 2006). GII is epidemiologically the most common genogroup causing 73 % of all reported HuNoV outbreaks from 1997 to 2000 in the U.S. (Fankhauser et al 2002). Human norovirus cannot replicate outside of a human host, but virus particles are relatively resistant and persistent in the environment and foods.

Clinical symptoms of HuNoV include diarrhea, vomiting, nausea, fever, fatigue, and stomach pain (Teunis et al. 2008). The infection is generally self-limiting but severe outcomes may result among elderly and immunocompromised patients. The infectious dose of HuNoV is very low, estimated at 10–2800 virus particles (Atmar et al. 2014; Patel et al. 2009; Teunis et al 2008). This means that HuNoV is extremely infectious and that even a small amount of contamination has the potential to cause illness. Human norovirus can cause acute gastroenteritis, mild gastroenteritis, and asymptomatic infection. It has been determined that ~20 % of HuNoV infected people are asymptomatic (Moe 2009) and that quantities of HuNoV present in the stools of asymptomatic individuals were similar to those in the stools of symptomatic individuals (Ozawa et al. 2007). This is important because asymptomatic carriers may handle or prepare foods, thus posing a potential health risk to the public. The exact impact of HuNoV asymptomatic carriers is not well understood for the reasons stated above. The following short case descriptions focus on the role that infected food handlers have played in HuNoV outbreaks at the point of service.

2.1. Case Study 1

In early September 2005, several cases of gastroenteritis were reported among rafters on unrelated trips on the Colorado River (Malek et al. 2009). Initially, it was determined that one supplier had provided delicatessen meat and cheese to all affected trips. On September 12, these items were withdrawn and an epidemiologic investigation was launched by the CDC. All rafters who reported diarrhea or vomiting on trips from August 14 to September 19 were included in the investigation, with an affected trip being defined as a rafting trip with ≥3 persons reporting acute gastroenteritis. Preliminary analysis limited the outbreak to 12 trips operated by five rafting companies with a total of 137 ill rafters, and also showed that the onset of symptoms generally occurred ≤72 h after the trip launch, which is consistent with the incubation period of HuNoV.

Common factors including transportation, hotel accommodation, guides or rafts, and drinking water, were ruled out. However, it was revealed that the five rafting companies purchased foods from three food suppliers. A case–control study was performed to identify the specific connection between outbreak illnesses and foods consumed; the evidence strongly suggested that launch day (the first day of rafting) was the common source of infection. The case definition was then further restricted to illnesses among rafters who reported symptoms of acute gastroenteritis during the first 72 h of their rafting trips. The

controls were defined as rafters on affected trips who never reported illness or those who became ill >3 days after trip launch. Data on symptoms, onset date and duration of illness, contact with ill rafters, contact with participants from other rafting trips, as well as food items eaten on the first day of the trip were collected via telephone interviews. The facility of the food supplier who provided delicatessen meats and cheese was inspected. Meat items including beef, chicken, ham, pepperoni, and turkey, as well as environmental samples were collected. In addition, stool samples were collected and analyzed from four ill rafters who participated in four different trips and five pooled samples from communal toilets used on one of these trips and on two other rafting trips.

A case control study revealed statistically significant association with delicatessen meats consumed on launch day. Two of the four individual stool samples from persons from four different trips were collected within a few days after resolution of symptoms and were found positive for HuNoV. Three of five communal toilet samples were also virus positive. All sequences were identical and belonged to the GII.4 genotype. The leftover delicatessen meat, as well as chicken and beef prepared in this batch, were also positive for HuNoV. Importantly, all the delicatessen meat consumed by the ill rafters was determined to have originated from the same batch prepared by a single employee on August 15 and 16 who used bare hands for machine-slicing and vacuum-packaging. This person had symptoms of diarrhea and vomiting from August 13–14, but recovered by the night of August 14, and returned to work on August 15 without requesting sick leave. More than 90 % of this batch of food was delivered to the five affected rafting companies on August 18–23. Therefore, this HuNoV outbreak was determined to be associated with ready-to-eat delicatessen meats contaminated by a food handler who had returned to work a day after recovering from a diarrheal illness.

2.2. Case Study 2

Another outbreak investigation started in a Texas hospital where 23 students from a local university experienced acute gastroenteritis on March 10 and 11, 1998 (Daniels et al. 2000). After interviews with selected ill students, matched and unmatched case–control studies were conducted using a questionnaire. The matched case–control study was first used to obtain information on specific foods eaten from March 4–10; and the unmatched case–control study further analyzed risks associated with food items previously identified. In addition to stool samples, food samples from the deli bar (ham sliced on March 9 and 10, turkey and salami sliced on March 10, and lettuce salad made on March 12) were collected for virus analysis. The case–control studies identified that the illnesses were more likely associated with foods served at the deli bar during lunch and dinner on March 9 and during lunch on March 10. An employee, who prepared the deli ham for sandwiches on March 9 for use in the meals (lunch and dinner that day and lunch for March 10), was suspected. She reported that her infant had been sick with watery diarrhea since March 7, which was 2 days before she prepared food for the implicated meals.

Fifty percent of ill students had HuNoV present in stool samples, and only ham, of all the remaining foods, tested positive. Importantly, the stool sample from the food handler's ill infant was also HuNoV positive with an identical sequence. This study reinforces the fact that food items may be easily contaminated by a food handler whose household member is sick.

2.3. Case Study 3

Food handlers were also involved in an outbreak at a residential summer camp during July 2005 in the province of Barcelona (northeast of Spain) (Barrabeig et al. 2010). There were 89 people in this summer camp including 72 children, 8 monitors, 6 employees, and 3 food handlers. On the night of July 14, 50 children developed symptoms of nausea, vomiting and abdominal pain; and only one of the three food handlers displayed these symptoms. All infected people ate the lunch on July 13 containing paella and/or round of beef. An epidemiological investigation was initiated among people with symptoms that were exposed to these foods. The suspected foods (paella and round of beef) prepared on July 13 in addition to stool samples from infected people and food handlers were collected. All samples were analyzed by RT-PCR to identify the causative pathogen; HuNoV GII.2 was detected in six stool samples. An asymptomatic food handler was also positive for the same genotype of HuNoV. However, HuNoV was not detected in the suspected food and no food was found to be significantly associated with infection following epidemiological investigation.

Although statistical analysis failed to show a connection between the two foods and HuNoV illness in this outbreak, beef remained a possible vehicle, since all infected people ate beef and paella or only beef, and there were four sick persons who ate beef but not paella. Importantly, the asymptomatic food handler whose stool sample was positive for HuNoV denied having any symptoms before and after the suspected lunch. The food handler did not consume the foods, but prepared, cut and served the beef. In addition, this food handler did not live in the same community as the outbreak patients. Since genotype GII.2 is not a very common genotype circulating in the outbreak community, other possible means of contamination could be ruled out. Even though there was no definitive evidence, this epidemiological investigation suggested that the asymptomatic food handler was probably the source of infection.

2.4. Prevention and Control

These three case studies indicate the ease with which foods can become contaminated through infected food handlers during preparation. It is crucial that food handlers returning from work after resolution of symptoms realize that they may still shed the virus. Food handlers must always use good personal hygiene and consideration should also be given to the ease in which employees can report illness and request sick leave. The current Food Code has restrictions to exclude food handlers for at least 24 h after resolution of symptoms associated with HuNoV and they are only allowed to work on a

restricted basis 24 h after symptoms resolve in a food establishment that does not serve a highly susceptible population (US FDA Food Code 2009: 2–201.12). Otherwise, the food handlers restricted from working in the food establishment serving a highly susceptible population, must remain restricted until they are medically cleared or otherwise meet safety criteria. It may be important to revise illness-related work-exclusion policies including paid leave for sick employees and increasing food handlers' awareness of food safety and disease transmission. Bare-hand contact with foods may pose risks and hence the use of gloves and stringent hand-hygiene requirements are necessary to reduce viral contamination. In addition, follow-up processing steps may help to inactivate pathogens present in foods.

3. ROTAVIRUS

Rotavirus belongs to the family *Reoviridae* and is the most common cause of severe diarrhea in infants and young children worldwide. In the U.S., rotavirus is the second most common cause of gastroenteritis since the successful introduction of rotavirus vaccine in 2006 (Parashar et al. 1998; Payne et al. 2013; Tate et al. 2011). Rotavirus infection occurs most frequently during the winter season from the months of November to April (Kapikian and Chanock 1996; Nakajima et al. 2001). The segmented, double-stranded RNA of the virus makes it genetically variable, often resulting in the creation of new strains during mixed infections. There are seven distinct antigenic groups of rotaviruses (from A to G) of which group A is most commonly associated with human illness. Two antigens can induce neutralizing antibodies: one present on the surface of the rotavirus outer capsid, which determines the G serotype and the other on the spike protein, which determines the P serotype (Estes 1996). Interestingly, circulation of five common strains (P[8]G1; P[8]G3; P[8]G4; P[4]G2; and P[8]G9) is more dominant (Gentsch et al. 1996; Yen et al. 2011). However, prevalence varies in different regions, mostly related to human activity, climate, and environmental conditions. Rotavirus can be easily transmitted from person to person via the fecal-oral route. The typical symptoms of rotavirus infection include watery diarrhea, anorexia, vomiting, and dehydration and infection may also be asymptomatic.

Generally, immunity to rotavirus develops in childhood and the infection is not common in adults although outbreaks have been reported in adults (Anderson and Weber 2004; Nakajima et al. 2001). A large number of rotavirus-associated outbreaks were linked directly to water, which is coincidently the ingredient often overlooked in foodborne outbreaks, along with ice made from contaminated water. Rotavirus has been detected in many foods, such as shellfish, vegetables, and sewage effluents, as described in the following case studies.

3.1. Case Study 1

On May 2, 2007, an outbreak of rotavirus was reported among children within a mother- and-child sanatorium in Germany (Mayr et al. 2009). At least six children were sick with symptoms of vomiting and/or diarrhea with or without a fever. Three children tested positive for rotavirus. The child who first reported illness had vomited in the dining hall on April 21 (day 1), and two other children started to display symptoms on days 3, 6, 8, and 10. Following that, a second wave of infection occurred with a child showing signs of illness on days 17, 19, 20, 22, 24, and 31. In addition, both parents of two siblings fell ill on days 6 and 8, but food handlers in the kitchen did not report illness. It is possible that other affected persons were not included in the epidemiological investigation due to a high fluctuation in the number of guests. After the second wave of infection started, 74 food samples from the sanatorium kitchen including soups, sauces, meat, vegetables, desserts, bread, sausages, and cheese were collected on day 21 for analysis. The food samples included were mainly those that had been served from days 9 to 16 of the outbreak. Rotavirus was detected in samples of potato stew and the sequence was 100 % identical with that obtained from one of the infected children's stool samples indicating that rotavirus may have been transmitted via the potato stew prepared on day 15. According to the timeline of infections, it is likely that the potato stew was contaminated on day 15 and was the causative agent of the second wave of infection. Since rotavirus is not resistant to heating, this indicates some improper handling behavior in the kitchen. Unfortunately, no environmental samples were collected during the epidemiological investigation, making it difficult to determine the exact route of virus transfer. No information was provided on how the viruses were transferred and spread to the kitchen area or other parts of the sanatorium. The contamination may have involved asymptomatic food handlers as they did not report any illness, or the virus may have been transferred during the first episode of vomiting that occurred in the dining hall.

3.2. Case Study 2

Another rotavirus-associated outbreak occurred in a dining hall on a university campus in the District of Columbia (DC) where foods including tuna and chicken salad sandwiches were involved (Fletcher et al. 2001). Beginning on 29th March, 2000, an increased number of students reported being sick with acute gastroenteritis. The local health department was notified, which launched an outbreak investigation. A case–control study was conducted via telephone interviews with students who reported illness. Additional ill students were identified during interviews and healthy controls were selected randomly from the university registry of students residing on campus. A case of acute gastroenteritis was defined as three or more episodes of diarrhea and/or two or more episodes of vomiting within a 24 h period with onset on or after

March 20. Information on food history, residence and dining hall, source of water, use of public access facilities on campus, and attendance at social events was collected from controls and case-patients. In addition, the employees working in the dining hall were interviewed; six employees reported illness. Stool samples from six sick students and 21 employees from dining hall were collected from March 29 to April 10. All stool samples were negative for bacterial and parasitic pathogens as well as for HuNoV. Group A rotavirus was detected in 9 of 27 samples (including four from students and three from employees) by electron microscopy, enzyme immunoassay, and RT-PCR. The rotavirus genotype was P[4]G2, which was not previously seen in previous outbreaks in the community. Two of the three employees that tested positive were line cooks; they reported having symptoms of gastroenteritis. The third virus-positive employee was a deli server who reported no illness.

During this investigation the number of cases among students rapidly increased after March 27, reaching a peak of 19 cases on March 31, followed by a gradual decrease. This trend indicated that the outbreak could have been caused by contaminated sandwiches served at the dining hall during the first week, which then spread from person-to-person during the following week. However, there is no definitive evidence to suggest that the foods were contaminated by the employees testing rotavirus positive before this outbreak occurred.

3.3. Case Study 3

A group of 11 people reported illness on April 24, 2000 after having dined together at a restaurant on April 17 (Japan Ministry of Health and Welfare and National Institute of Infectious Diseases 2000). Epidemiological investigation revealed that 22 of 59 persons who had meals at the restaurant on April 17 developed gastroenteritis. The symptoms consisted of diarrhea, abdominal pain, fever, vomiting, and nausea. Stool samples were collected from 12 patients and three food employees. Group A rotavirus was detected in nine stool samples (seven from patients and two from employees).

3.4. Prevention and Control

Rotavirus was the cause of foodborne illness in people of all ages in the cases described above. In order to limit epidemic gastroenteritis and reduce the number of illnesses, preventive measures are needed. Previously, a community-based prospective cohort study with nested case–control study identified risk factors that were most likely to cause rotavirus infection. Contact with patients and inadequate food-handling hygiene in the household were strongly associated with rotavirus infection (de Wit et al. 2003). In another study, 43 % of rotavirus inoculated on human fingers could persist for up to 60 min (Mayr et al. 2009). Therefore, similar to guidance for reducing HuNoV, good hand washing is essential for rotavirus prevention. In addition, the rotavirus vaccine is an alternative way to prevent infection.

4. HEPATITIS A VIRUS

Hepatitis A (HAV) is a viral disease of the liver, causing mild to severe illness worldwide. The HAV is a non-enveloped, single stranded RNA virus (7.5 kb) (Cohen et al. 1987), which belongs to the family *Picornaviridae* and is the only member of the *Hepatovirus* genus. It is also the most common agent causing acute liver disease worldwide (Vaughan et al. 2014) but does not cause the chronic liver disease that often occurs with hepatitis B and hepatitis C infections (WHO 2013). The HAV is one of the most frequent causes of foodborne illness due to its presence and persistence in the environment, as well as its ability to survive food processing mechanisms typically used to control bacterial pathogens (WHO 2013). Transmission of the virus occurs via the fecal-oral route, via person-to-person contact, or after an uninfected or unvaccinated person consumes food or water contaminated with the feces of an infected person (CDC 2009; WHO 2013). Waterborne outbreaks tend to be less frequent and are typically associated with a sewage contamination event or inadequately treated drinking or recreational water (WHO 2013). Although rare, HAV can also be transmitted via blood transfusion or blood products originating from infected donors during viremia (de Paula 2012). However, the disease is typically associated with poor water quality, poor sanitation, and poor personal hygiene. Symptoms of HAV infection are usually more severe in older children and adults and may include fever, malaise, anorexia, diarrhea, nausea, abdominal pain, dark-colored urine and jaundice. The incubation period is typically 14–28 days (WHO 2013).

According to the World Health Organization, an estimated 1.4 million cases of HAV occur worldwide every year with cyclic recurrences leading to significant economic and social consequences (WHO 2013). Notably, the impact of HAV outbreaks in food establishments can be devastating. The number of HAV infections varies greatly from country to country and depends on various socioeconomic factors including water quality and sanitation practices (Vaughan et al. 2014).

Areas with high incidence of HAV infection include developing countries where sanitation is poor. A majority of children in these areas (90 %) are infected with the virus by the age of 10 although they are typically asymptomatic (WHO 2013; Munne et al. 2008). As a result, the occurrence of HAV epidemics and outbreaks is relatively uncommon in these areas because adults are generally immune (Wasley et al. 2006; WHO 2013). Developing countries with transitional economies, where sanitary conditions are variable, usually show lower infection rates in children and intermediate levels of infection in the general population. However, because they are not infected during childhood, adolescents and adults in these areas are more susceptible to severe HAV infection and large outbreaks are more likely to occur (WHO 2013). Low levels of infection are typically seen in developed countries where sanitation and hygiene practices are good. High risk groups in developed countries

include injection drug users, men who have sex with other men, people who travel to endemic areas, and people living in isolated populations such as closed religious communities (WHO 2013).

Between 1994 and 2004, more than 250 HAV outbreaks were reported in the U.S. including 80 different foodborne outbreaks (Grieg et al. 2007). In industrialized countries and countries in a state of economic transition where endemicity is low and the number of susceptible adults is high, foodborne outbreaks associated with HAV remain a major public health problem (Vaughan et al. 2014).

4.1. Case Study 1

A major outbreak associated with orange juice occurred among tourists in Egypt (Frank et al. 2007). In August of 2004, disease surveillance in Germany showed an increase in HAV infections in tourists who recently travelled to Egypt—a place where HAV is highly endemic (Darwish et al. 1996; Ramia 1986). The majority of case patients had stayed at the same hotel (hotel X), where the outbreak investigation took place. The case definition included persons with laboratory evidence of recent HAV infection (presence of anti-HAV IgM) who stayed at hotel X after June 1, 2004, as well as persons who were current hotel guests at hotel X with symptoms such as jaundice or elevated liver enzyme levels without laboratory confirmation. The epidemiological investigation also included a case–control study among guests of hotel X >17 years of age residing in three German states. Investigators identified the "minimum period of transmission" (MPT) as the time period between the earliest arriving case patient's last day at the hotel and the latest arriving case-patients first day at hotel X. Case-patients were selected from this line listing. Control patients were healthy control guests who stayed at hotel X during the MPT who had neither been vaccinated for HAV, nor previously infected with HAV. A standardized questionnaire was administered over the phone. Investigators collected information during phone interviews including demographics, food and drink consumption, participation in day trips, and recreational activities during the person's stay at hotel X. An environmental investigation also took place where all hotel employees and food suppliers were tested for HAV.

This outbreak lasted from July 10–September 8, 2004 with a total of 351 case patients identified. A total of 271 primary and 7 secondary cases were identified in Germany with an additional 60 primary infections reported in eight other European countries. Clinical HAV infection developed in 97 % of primary cases. The age range of case patients was 2–67 years of age (median age of 34) and 54 % of case patients were males. Hospitalization occurred in 47 % of case patients and risk for hospitalization increased with increasing age. The MPT lasted from June 24–July 23, where case patients stayed at hotel X from 6 to 21 days. The majority of case patients (70 %) stayed 13 days or longer. Approximately 52 % of case patients had stayed together at the hotel on any single day. Statistical analysis included 69 HAV case patients and 36 controls

(there was a 60 % response rate among the 115 case patients in the three states of Germany). There was no significant difference among case patients in terms of age, sex, recreational activities, consumption of foods or behavioral characteristics. However, case patients were significantly more likely to have consumed orange juice served at breakfast buffet (82 %) than the controls (64 %) (OR 2.6, 95 % CI). A dose-dependent relationship became evident between the number of days of orange juice consumption and development of HAV infection. The median number of days of consumption of orange juice among case patients was 11 days, whereas controls only consumed orange juice for a median of 5 days. During the environmental investigation, none of the hotel employees tested positive for HAV IgM. There was minimal fluctuation among hotel staff, leading investigators to conclude that it was unlikely that a HAV-positive employee was missed. At the orange juice production facility, investigators reported significant hygiene problems. They also reported that the orange juice was not pasteurized.

In summary, this outbreak demonstrates the risk for non-immune travelers visiting an HAV-endemic area. No sources of the outbreak were identified other than orange juice consumption, of which higher doses of orange juice consumption were correlated with increased risk of HAV infection. It was concluded that the orange juice was contaminated most likely by an infected food handler during production or by contact with fruit or machinery that had been washed with sewage-contaminated water. Although fruit juices are rarely associated with HAV outbreaks, the virus is very resistant to acids (Siegl et al. 1984) and could potentially survive long periods of time in orange juice. Interestingly, orange juice was consumed by 60 % of healthy controls; this could be explained in part by the possible fluctuating virus concentration within the juice, resulting in varying degrees of infectious viral particles present in the juice during the 4-week period. A contaminated lot of juice could have slowly been mixed in and out with other lots. In addition, some control cases may have been immune to HAV but did not know it.

4.2. Control and Prevention

Outbreaks of HAV have taught the public and the scientific community the importance of immunization, particularly in endemic areas as well as persons who plan to travel to endemic areas. According to the World Health Organization, nearly 100 % of people develop protective levels of antibodies to the virus within 1 month after a single dose of the vaccine and millions of people worldwide have been vaccinated with no serious adverse effects. People at an increased risk of contracting HAV are especially encouraged to get vaccinated, including persons travelling to endemic areas, men who have sex with men and persons with chronic liver disease. A vaccination program can help control community-wide outbreaks; however, they are most successful in small communities (WHO 2013). It is recommended that vaccination programs be accompanied by health education programs to improve

sanitation, personal hygiene, and food safety practices (WHO 2013). As we have learned from the above outbreak, secondary infections can occur, indicating the importance of improved personal hygiene and hand washing practices. In endemic areas, adequate supplies of drinking water and proper disposal of sewage within communities should be practiced to prevent spread of HAV. Regular hand-washing with safe water should also be practiced within these communities.

5. AICHIVIRUS

Aichiviruses are small non-enveloped viruses with a positive sense RNA genome. These viruses belong to the *Picornaviridae* family and *Kobuvirus* genus (Lodder et al. 2013). The first incidence of Aichi virus in humans was reported in 1989 in Japan and was associated with the consumption of oysters (Yamashita et al. 1991). Symptoms of Aichivirus infection include diarrhea, abdominal pain, nausea, vomiting, and fever (Yamashita et al. 1991, 2001). Low incidence of Aichivirus has been found in patients suffering from gastrointestinal illness around the world although the seroprevalence is high in the community (Lodder et al. 2013; Pham et al. 2007; Yang et al. 2009; Ambert-Balay et al. 2008; Jonsson et al. 2012; Kaikkonen et al. 2010; Sdiri-Loulizi et al. 2010; Verma et al. 2011). Serological studies show that up to 95 % of the world's human population has been exposed to the virus by the age of 40 (Reuter et al. 2011).

Currently, little is known about the epidemiology and mechanism of transmission of Aichivirus (Lodder et al. 2013). There is some indication that Aichiviruses are spread via the fecal-oral route since they have been detected in sewage, surface waters, and river waters in various countries including Tunisia (Sdiri-Loulizi et al. 2010), Venezuela (Alcalá et al. 2010), Japan (Kitajima et al. 2011), and Norway (Lodder et al. 2013). High levels of Aichiviruses were found in sewage and water samples from Japan and Norway. In Japan 100 % (12/12) of raw sewage, 92 % (11/12) of treated sewage, and 60 % (36/60) of surface water samples tested positive for Aichivirus (Kitajima et al. 2011). In Norway, 100 % (14/14) of sewage samples and 85 % (12/14) of surface water samples tested positive for Aichivirus (Lodder et al. 2013). Human feces containing Aichi virus can contaminate surface waters directly or through treated or untreated sewage (Lodder et al. 2013). It is hypothesized that humans are exposed to the virus via consumption of untreated surface waters, swimming in contaminated recreational waters, and consumption of raw shellfish (Lodder et al. 2013). The types of viruses circulating in sewage waters have been shown to correlate with those circulating in symptomatic or asymptomatic human populations (Lodder et al. 2012).

More studies on the prevalence of Aichiviruses in the population and the environment are necessary to determine their epidemiology and pathogene-

sis so effective disease prevention strategies can be developed and implemented. It is also suggested that samples be tested for Aichi virus in cases where the cause of gastrointestinal illness cannot be determined (Lodder et al. 2013). The role of Aichiviruses in disease development among elderly and immunocompromised individuals should also be determined as Aichiviruses have been detected in hospitalized elderly patients experiencing diarrhea in Sweden (Jonsson et al. 2012). Studies should also be conducted to determine the prevalence of Aichivirus in the environment, specifically surface waters and sewage, in various parts of the world. These data could then be correlated with clinical prevalence or surveillance data from gastrointestinal outbreaks (Lodder et al. 2013).

6. HEPATITIS E VIRUS

Hepatitis E virus (HEV) is a member of the *Hepeviridae* family and is composed of single stranded, non-enveloped, positive-sense RNA with a length of approximately 7.5 kb (Faramawi et al. 2011; Reyes et al. 1990). Models derived from epidemiological and clinical studies project an annual burden of about 3.4 million symptomatic cases, 70,000 deaths and 3000 stillbirths (Perez-Gracia et al. 2014). The HEV infection is usually self-limiting and resolves within 4–6 weeks but can develop into clinical hepatitis (acute liver failure) in young and middle aged adults, pregnant women, and immunocompromised individuals. Symptoms typically last 1–2 weeks and include jaundice, loss of appetite, enlarged liver (hepatomegaly), abdominal pain and tenderness, nausea, vomiting, and fever.

The HEV occurs worldwide with the highest prevalence in Southern and Eastern Asia (WHO 2013). It is an emerging zoonotic infection that has increased in importance in developed countries such as the U.S. where 1 in 5 people are now seropositive for viral antibodies (Daniels et al. 2007; Faramawi et al. 2011; Jacobsen and Koopman 2004; Kuniholm et al. 2009). Humans are considered to be the main hosts for HEV although antibodies to the virus have been detected in several other animal species including primates (WHO 2013). Four epidemiologically distinct genotypes of HEV are known. Genotypes 1 and 2 are isolated from humans and are found more often in developing countries. Genotypes 3 and 4 are isolated from humans and animals and are more commonly found in developed countries (WHO 2013). Seroprevalence is the highest in regions with poor sanitation, which increases the risk for transmission of the virus (WHO 2013). Genotype 1 is predominantly found in North Africa and Central and Southeast Asia while genotype 2 is found in Mexico and West Africa (Perez-Gracia et al. 2014). Genotype 3 is found predominantly in North and South America, Europe and Japan and genotype 4 is found mostly in China (Perez-Gracia et al. 2014).

The HEV is spread via the fecal-oral route through contaminated water and undercooked meat (WHO 2013; Faramawi et al. 2011). The main transmission route in developing countries is the consumption of water contaminated with fecal matter, often associated with flooding episodes where rain water is mixed with human sewage (Perez-Gracia et al. 2014). In developed countries transmission more likely occurs via the consumption of raw meat of infected animals or consumption of raw produce washed with contaminated water (Perez-Gracia et al. 2014). A U.S. study revealed that approximately 11 % of pork obtained from various butchers was HEV positive (Feagins et al. 2008). The virus is environmentally stable and can easily persist in uncooked, undercooked, and frozen foods. A unique feature of HEV, and an important reason to assess its transmission and pathogenesis relative to food safety, is the relatively high mortality associated with the virus in high-risk populations. Additional reservoirs of HEV include poultry, swine, boar and deer (Perez-Gracia et al. 2014). A vaccine to prevent HEV infections has been produced and licensed in China but is not yet available globally (WHO 2013).

6.1. Case Study 1

A well-documented outbreak of HEV occurred in Northern Uganda (Howard et al. 2010). In October 2007, several cases of jaundice were reported by the health facility of a rural internally displaced persons (IDP) camp in the sub-county of Madi Opei in the northern Uganda district of Kitgum. These cases were confirmed to be due to genotype 1 HEV. By March of 2009, cases of HEV were being reported in additional sub-counties of the district including the primary site of the outbreak investigation that was taking place in Paloga. By June 2008, there were a total of 3350 suspected cases and 67 deaths reported as a result of the outbreak. By the end of 2008, there were more than 9500 reported cases and 148 deaths associated with the outbreak. A case control study and an environmental investigation were conducted from June 17–24 of 2008 to identify risk factors and potential sources of infection.

The case definition was defined as any individual with jaundice, as confirmed by a physician; at least one accompanying symptom, including fatigue, anorexia, abdominal pain, arthralgia, fever or headache; and serological evidence of HEV infection. The control was defined as a person who had no symptoms or serological evidence of HEV infection. Controls were matched to cases based on age group of case patients and location of primary residence. The study included 60 case patients and 180 controls based on the assumption of a 3:1 ratio of controls to cases and an odds ratio (OR) detection limit of 2.5. Participants filled out a structured questionnaire with a requested recall period of 8 weeks (taking into account the incubation period of HEV infection) and gave blood samples for HEV testing. Laboratory testing was performed at the Hepatitis Reference Laboratory at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA.

Environmental samples were collected from Paloga and other nearby sub-counties that were affected by the outbreak in the Kitgum District. Water samples were collected from boreholes prior to chlorination treatment.

A convenience sample of five households with a current or recent case of jaundice was also collected from household water storage vessels (unchlorinated). Water was also collected from three streams and one pond that were reportedly used by local residents for bathing.

A majority of cases (59.8 %) was between the ages of 15 and 45 years and their primary residence within the last 8 weeks had been in Paloga (80.7 %). Demographics including gender, household size, and education level were not found to be significantly associated with the disease. However, the incidence of disease was significantly associated ($P<0.20$) with bathing in the local river, storing drinking water in a large-mouthed container, preparation of rats for consumption, consumption of homemade alcoholic brew made from river water, washing hands in a shared basin, eating from a shared plate of food, promiscuous defecation, and improper disposal of children's feces. Investigators were surprised to find that specific factors, such as having recently drank water from the river, having a borehole as a primary source of drinking water, having pigs or goats at home, having chlorine tablets distributed with drinking water, and having been to a funeral where communal hand washing was common, were not associated with the incidence of disease. According to statistical analysis using a conditional logistic regression model the two most significant factors ($P<0.05$) associated with the disease were storage of drinking water in a large-mouthed container (AOR = 2.8; CI = 95 %) and hand washing in a common basin (AOR = 1.9; CI = 95 %). Viral RNA was detected in water from two of four surface water sources, including one stream in Paloga. One of two water samples that had been used to rinse the hands of patients, who were currently displaying symptoms of jaundice, was also positive. Viral RNA was not detected in any of the 15 drinking water samples that were collected in this study.

This was the largest HEV outbreak reported in a displaced population. It was concluded that HEV transmission could occur at the household level or via person-to-person contact, particularly in crowded living conditions. A point source was not identified for this large outbreak but epidemiological and environmental data indicated that improper water storage and hand washing habits played a large role in the incidence of disease. The authors concluded that, to prevent future epidemics, basic control measures such as water decontamination and provision of proper sanitation resources needed to be implemented at the community level. In addition, supplies, services, and education related to personal and household hygiene should be made available. A safe and effective HEV vaccine would be useful for (1) people living in developing countries where pre-existing immunity among exposed populations is believed to be non-existent and (2) for high-risk populations e.g., pregnant women.

6.2. Case Study 2

A well document outbreak of HEV Genotype 4 occurred in Italy in 2011 (Garbuglia et al. 2013). Acute HEV infection was detected in five patients admitted to hospitals in the cities of Lazio, Rome, and Latina. The HEV infection was confirmed on the basis of clinical signs of acute hepatitis and

laboratory confirmation via the detection of IgG and IgM antibodies by immunoassays. The health authorities conducted structured interviews with each case patient to determine their travel history and other risk factors. None of the case patients reported travel to endemic areas and a common source could not be identified. Serum samples from patients were screened for HEV RNA by reverse transcriptase PCR, which identified a HEV genotype 4. The virus showed high genetic similarity among patients (99.2 %) and to previously isolated HEV strains, including those isolated from swine and humans in China. However, the similarity of the strain from this outbreak to strains in recent autochthonous HEV genotype infections in Europe was found to be relatively low (73.5 %).

Due to the high genetic similarity among HEV strains and the lack of previous travel of case patients to endemic areas, the authors suggested that a point-source was responsible for this outbreak as opposed to person-to-person transmission. The epidemiologic information did not help investigators identify the source but did allow them to exclude other causes including direct contact among cases and parenteral transmission. It was concluded that consumption of contaminated food was probably responsible for this outbreak. The isolates from this study had the highest genetic similarity to sub-genotype 4 strains of swine originating from China, which suggests a possible zoonotic origin through either direct contact or consumption of raw pork products or indirect contact via consumption of water contaminated with animal feces. Although not as likely, authors could also not rule out other possible sources of infection including consumption of contaminated food originating abroad and direct introduction of the virus through infected immigrants from China or other countries in Asia. Molecular characterization of HEV isolates that cause outbreaks in Europe is needed to implement epidemiologic mapping of infection that may help identify potential sources of HEV.

6.3. Prevention and Control

When an outbreak occurs, the WHO guidelines for epidemic measures include determining the mode of transmission, identifying the population specifically exposed to increased risk of infection, eliminating a common source of infection, and improving sanitary and hygienic practices to eliminate fecal contamination of food and water (WHO 2013). In developing countries, the risk of infection and transmission can be reduced by maintaining quality standards for public water supplies as well as establishing proper disposal systems to eliminate sanitary waste. Hand washing should be performed with safe water before preparing food. Drinking water of unknown purity or origin should be avoided. Individuals should also avoid eating any uncooked meat or shellfish. They should also avoid eating uncooked fruits or vegetables that are peeled or prepared by people living or traveling in endemic areas (WHO 2013). The WHO also recognizes World Hepatitis Day on 28 July every year to increase awareness and understanding of viral hepatitis.

7. SUMMARY

While the primary route of transmission of enteric viruses like HuNoV, rotavirus and hepatitis A virus is fecal-oral, community transmission may also occur through infected individuals and asymptomatic food handlers. Transmission tends to occur most frequently during cold seasons when people are indoors and are in more frequent contact with each other, as well as in places where people are housed in close quarters such as dormitories, nursing homes, hotels and resorts. Unfortunately, adequate virus testing within the food industry is limited by cost as well as by the lack of advanced rapid detection technology; however, the time for testing and costs are likely to be lower in the future. The symptoms caused by foodborne viruses occur hours or even days post exposure making trace-back more difficult. Investigators rely on good epidemiology to detect the virus and often the test results of food and stool samples lead to inconclusive results. Emerging foodborne viruses like Aichivirus and HEV have caused several outbreaks outside of the United States and their footprints continue to grow. The evidence of zoonosis surrounding HEV has also drawn a great deal of attention. The case studies described in this chapter demonstrate the role food handlers can play in food contamination. The above case studies are based on passive surveillance; active surveillance would identify potential trends of outbreaks and illnesses associated with foodborne pathogens.

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CHAPTER 9

Methods for Virus Recovery from Foods

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1. INTRODUCTION

Viruses are increasingly being recognized as a major cause of foodborne illnesses and outbreaks. It is estimated that more than half of the foodborne illnesses in the United States are caused by viruses (Scallan et al. 2011). In 2012, viruses were responsible for 14 % of all reported foodborne outbreaks in the European Union (EFSA 2014a). Human norovirus (NoV) and hepatitis virus type A (HAV) are most commonly transmitted by food, although other viruses such as astrovirus, sapovirus, enteric adenovirus, parvovirus, and Aichi virus are also important (Gallimore et al. 2005; Le Guyader et al. 2008). The most common foods involved in these outbreaks are shellfish and fresh or minimally processed fruits and vegetables. Oysters are well recognized as the main vectors of environmentally transmitted NoVs and disease outbreaks linked to oyster consumption have been commonly observed (Shieh, et al. 2007; Yu et al. 2015). Also, random sampling at market places and oyster farms in Europe and the U.S. revealed the presence of NoV in 5–55 % of tested samples (Boxman et al. 2006; Costantini et al. 2006; Gentry et al. 2009). Several types of fresh produce (e.g. green vegetable salads, lettuce, raspberries, strawberries, cabbage, kimchi, pomegranate seeds, and raw frozen fruit mixes) have been responsible for disease outbreaks after being contaminated by polluted water or virus-infected food handlers (Table 9.1). Between 1998 and 2005, NoV-contaminated green salads, lettuce, fruits, and vegetables contributed to 24, 5.1, 3.2, and 2.3 % of all produce-based outbreaks, respectively (Dewaal and Bhuiya 2007).

Viruses are usually found in food matrices in very low numbers because they cannot replicate in host-free environments including water and food matrices. Food matrices serve only as vehicles for virus transmission to humans. However, despite their low numbers in contaminated foods, viruses are still hazardous to consumers' health due to their low infective dose. For instance, as little as 18–1000 virus particles of NoV are enough to cause infection in a susceptible host (Teunis et al. 2008; Koo et al. 2010; CDC 2015a). The current scientific revolution in molecular techniques (e.g., PCR and RT-PCR) has made the detection, identification and titration of viruses in clinical samples easier than before. However, it is not easy to detect viruses in food matrices because:

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Table 9.1 Foodborne outbreaks of NoV and HAV transmitted by fresh produce from 2005 to 2015

Implicated food	*Virus (genotype)*	*Year and location (location of origin if international)*	*No. of cases*	*Reference*
Berries (frozen mixed)	HAV (IB)	2013, U.S.A. (Turkey)	162	CDC (2015b), Collier et al. (2014)
Berries (frozen mixed), fresh berries, mixed berry cake frozen berry mix cake	HAV (IA)	2013–2014, Italy, Ireland, the Netherlands, Norway, France, Germany, Sweden, U.K., Finland	1444	Chiapponi et al. (2014), ECDC (2014), EFSA (2014b), Guzman-Herrador et al. (2014), and Rizzo et al. (2013)
Cabbage kimchi	NoV (GI.3)	2011, Korea	451	Cho et al. (2014)
Lettuce (salad and soup)	NoV (GII.4)	2008, Portugal	16	Mesquita and Nascimento (2009)
Lettuce	NoV (GI and GII)	2010, Denmark (France)	260	Ethelberg et al. (2010)
Pomegranate seeds	HAV (IB)	2012, Canada (Egypt)	6	Swinkels et al. (2014)
Salad	NoV (GII.7)	2006, Austria	182	Schmid et al. (2007)
Salad (mixed)	NoV (GII.4)	2007, U.K.	34	Showell et al. (2007)
Salad	NoV (GII.6)	2007, U.K.	79	Vivancos et al. (2009)
Salad buffet vegetables	NoV (GI.3)	2007, Sweden	413	Zomer et al. (2010)
Salad vegetables	NoV (GII.4)	2007, Japan	23	Oogane et al. (2008)
Salad (dried radish)	NoV (GII.4)	2008, Korea	117	Yu et al. (2010)
Salad	NoV (GII.4)	2009, Germany	27	Wadl et al. (2010)
Frozen raspberries	NoV	2005, France	75	Cotterelle et al. (2005)
Frozen raspberries	NoV	2005, Denmark	466	Korsager et al. (2005)
Frozen raspberries	NoV	2006, Sweden	43	Hjertqvist et al. (2006)
Frozen raspberries	NoV	2006, Sweden (China)	43	Hjertqvist et al. (2006)
Frozen raspberries	NoV (GI.4)	2009, Finland (Poland)	200	Maunula et al. (2009)
Frozen raspberries	NoV (GII.4, GII.b, GII.7, GI.4)	2009, Finland (Poland)	900	Sarvikivi et al. (2012)
Frozen strawberries	HAV (IB)	2012–2013, Denmark, Finland, Norway, Sweden	103	Nordic Outbreak Investigation (2013), Lassen et al. (2013)
Frozen strawberries	NoV (GII.16/ II.13b; GI.9; GII.6)	2012, Germany (China)	11,000	Mäde et al. (2013) Bernard et al. (2014)
Vegetables	NoV	2009, Minnesota, USA	16	CDC (2015b)

1. Viruses are present in low numbers in foods and, unlike bacterial pathogens, their numbers cannot be increased by enrichment pre-steps because the viruses cannot replicate in the absence of a living host.
2. Hence, viruses need to be concentrated from large amounts of food sample to increase the probability of their detection. However, the complexity of food matrices makes it difficult to design a universal method of virus concentration/extraction that can be used to recover viruses from various food types. Hence, complicated, multistep and time consuming viral recovery strategies are often used.
3. Food matrices may contain PCR inhibitors (e.g. urea, polyphenolics, tannic acid, polysaccharides, pectin, melanin, xylan, collagen, myoglobin, hemoglobin, lactoferrin, immunoglobin G, and proteinases), which may lead to failure of virus detection by molecular methods (Goyal 2007; Schrader et al. 2012).
4. The most important foodborne viruses (NoV and HAV) either do not grow in cell cultures or are difficult to grow making it difficult to isolate them from various foods.

It is obvious that the successful detection of viruses in foods depends on the use of efficient strategies for virus recovery from various types of food. The attributes of an ideal method for virus extraction from food samples are as follows:

1. The method should be applicable to several different types of foods.
2. It should be simple, rapid, reproducible, and inexpensive.
3. The final sample volumes should be small so that they can be easily subjected to virus isolation in cell cultures and/or virus detection by molecular methods.
4. The final extract should not be cytotoxic to cell cultures.
5. The final extract should not contain substances that can inhibit PCR and RT-PCR reactions (Kittigul et al. 2015).
6. The method should be applicable to large quantities of foods so that small numbers of virus present in large amounts of food can be easily concentrated.

Thus, development of efficient virus extraction methods is of interest to the food safety community who can use it routinely for in-line quality assurance of food processes. It is of importance to food virology investigators to assess the risk of viruses in food, study the fate of viral pathogens from field to the fork, and investigate possible strategies for prevention of foodborne viral contamination. Several virus recovery methods have been developed and used and are still a subject of current researches. The differences among these methods depend on the food matrix and its characteristics. In this chapter, summarized explanation and comparisons between these methods will be addressed. Our focus will be on fresh produce only as the shellfish methods are discussed in a separate chapter in the book.

2. FOOD SAMPLING FOR VIRUS DETECTION

Selecting of a sampling procedure is very critical for any analytical procedure because it is the first step which affects significantly the reliability and certainty of the results obtained. It is even more important for food sampling because interpretation of test results about a large consignment of food is based on testing a relatively small sample from a given lot. If samples are improperly collected and mishandled or are not representative of the sampled lot, the laboratory results will be meaningless. Many important questions come to mind regarding adequate sampling e.g., (1) is certain weight/volume of a particular fruit or vegetable representative of the whole lot, (2) is one item of fruit or vegetable per crate representative, (3) how many and which leafs of a leafy produce are representative, (4) should the food item be analyzed whole or chopped, and (5) how many replicates are required?

In this regard, two general conditions need to be considered: (1) the sample taken for testing has to be randomly selected and (2) it should be representative of the whole bulk or batch of the tested food. In addition, the US Environmental Protection Agency (US EPA), the International Organization for Standardization (ISO), and the National Quality Assurance (QA) regulations must be followed. References to sampling for microbiology usually refer to economic and logistical considerations as other factors often limit the number, type, and location of samples to be tested (Murray 2002).

Although the viral load on fresh or minimally processed produce and on ready-to-eat foods may be low, it is still of concern because of the low infectious dose of foodborne viruses. Therefore, the methods for detection of viruses in food samples must have a high level of analytical sensitivity and specificity. There is, however, very little information in the literature, from the US EPA, in Bacteriological Analytical Manual of Food and Drug Administration (US FDA-BAM), the European Committee for Standardization (CEN), and in ISO guidelines regarding sampling procedures for the analysis of viruses in food. For bacteria, a 25 g sample was standardized as an adequate analytical unit-size for detecting a pathogen such as *Salmonella spp.* Also the analytical units can be composited (e.g., fifteen 25 g units can be pooled into a 375 g composite). In addition, an enrichment step must be performed prior to the detection procedure (FDA-BAM) thus increasing the chances of bacterial detection. However, sampling for virological analysis of food cannot follow the bacterial approach due to their low level of contamination, the lack of an enrichment step and the complexity of food matrices. Many of the published methods for the detection of viruses on fruit and vegetables use a 10–100 g sample size (Table 9.2) but there is no mention of how many 10–100 g samples need to be tested from a crate, from a field, or from a truckload of food to have a statistically representative sample (Bosch et al. 2011).

It is also not clearly understood if food samples should be whole or chopped before being analyzed. Since most viral contamination occurs from external

sources during spraying or irrigation, a critical factor affecting the decision to analyze whole or chopped vegetables would depend on the veracity of the claim that viruses can enter plants through root damage. For example, internal contamination of the leaves of tomato plants and green onions has been reported (Oron et al. 1995; Chancellor et al. 2006) but at a much lower level than that caused by external contamination (Carter 2005; Urbanucci et al. 2009). In certain specific cases (such as washed or ultraviolet-irradiated vegetables), the detection of viruses in internal parts of a plant can be used as a potential indicator of external contamination since internalized viruses would not be removed or inactivated by washing or ultraviolet irradiation (Carter 2005). In the case of foods implicated in viral disease outbreaks, the sampling would focus only on the particular batches of food consumed (Bosch et al. 2011). Obviously, sampling procedures vary depending on the type of food matrix and have to take the following into account: (1) the quantity of sample, (2) seasonality, (3) rainfall, and (4) probable amount of contamination or pollution (Bosch et al. 2011).

3. STRATEGIES FOR RECOVERY OF VIRUSES FROM FOODS

The old methods for detecting human enteric viruses in foods were based on inoculation of the food extracts into cell cultures that were susceptible to the virus of interest. However, because some enteric viruses cannot be grown in cell culture (e.g., human NoV), the use of the molecular technique of reverse transcription-PCR (RT-PCR; involving the amplification of conserved regions of the virus genome) has become a common way to detect viruses in foods. Although this technique is very sensitive and specific, it is unable to discriminate between infectious and non-infectious virus particles (Croci et al. 2008). However, recently two methods employing porcine mucin-coated magnetic beads and ethidium monoazide dye were developed and show promise in achieving this objective (Dancho et al. 2012; Moreno et al. 2015). In order to perform the molecular detection of viruses in foods, a general three-step strategy is followed in most published work. It consists of the following: (1) virus recovery, (2) extraction of the nucleic acid of the viral pathogen and (3) molecular detection of the viral RNA (Fig. 9.1). This chapter focuses only on the strategies for virus recovery from foods (Step 1) while the other two steps are addressed in other chapters of the book.

The type of method used for virus recovery from foods depends on the food composition and characteristics. In this regard, the foods can be classified into three distinct categories (Baert et al. 2008): (1) foods composed of carbohydrate and water, mainly fruits and vegetables, (2) fat- and protein-based foods such as ready-to-eat products, and (3) shellfish as a separate category because of accumulation and concentration of viruses in their digestive system

Table 9.2 Methods used for recovery of enteric viruses from fruits and vegetables

Food	*Sample size*	*Virus*	*Approach*	*Elution*
Lettuce Cabbage Celery Spinach	4.5–9.8 kg (chopped)	PV1[a] Adenovirus[a]	1. Elution 2. Clarification 3. Concentration (two steps)	Phosphate buffer pH 9 (24 L) with shaking 7–10 min
Lettuce	20 g (chopped)	Unknown	1. Elution 2. Clarification 3. Concentration	Glycine—NaOH buffer pH 8.8 (100 mL)
Frozen strawberry	25 g (homogenized)	Unknown	1. Elution 2. Clarification 3. Concentration	glycine—NaOH buffer pH 10.8 (100 mL) with homogenization for 5 min
Lettuce Celery Carrot Radish	20 g (chopped)	Simian rotavirus[a] (surrogate for RV)	1. Elution 2. Clarification	1. 3 % beef extract 2. 3 % tryptose phosphate broth 3. 0.05 M glycine buffer 4. Distilled water (50 mL) Three pH (8, 9, 10) were tested for each eluent. (Shaking for various times)

Clarification	*Concentration*	*Percent virus recovery*	*Notes*	*Reference*
Filtration through glass wool pretreated with Eagle basal maintenance medium containing 2 % fetal calf serum to prevent loss by virus adsorption	Two steps: **1. Filter adsorption** and elution. – pH adjusted to 3.5 – Filtration (glass fibers, melanin-impregnated paper, epoxy pleated cartridge filter) – Secondary elution by 3 % beef extract pH 9 **2. Organic flocculation** – 3 % beef extract pH 3.5 was used as a flocculent – Centrifugation 3000 × g – dissolved in phosphate buffer pH 9.0	58 % (mean recovery)	–	Ward et al. (1982)
Flocculation with CatFloc TL and filtration on Whatman filter paper or cellulose triacetate filter	Ultrafiltration using Amicon filter	–	–	Cliver et al. (1983)
Centrifugation after mixing with $MgCl_2$ and bentonite	Flocculation with 30 % PEG 20,000 followed by ultrafiltration	–	– Because of the acidity of the strawberries, unusually alkaline buffer (pH 10.8) was used. – $MgCl_2$ and bentonite were used to facilitate the separation of solids in centrifugation	
Filtration through 0.2 µm filter after neutralization to pH 7.2	No concentration	65 % Lettuce 49 % celery 34 % carrot 50 % radish (using eluent no. 1)	The best eluent was 3 % beef extract at pH 8. Non-leafy vegetables showed lower recovery. Elution time more than 5 min decreased the recovery	Badawy et al. (1985)

(continued)

Table 9.2 (continued)

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Delicatessen foods (method 1)	20–40 g	NoV GI[a] HAV[a]	1. Elution 2. Clarification 3. Concentration	3X Wash with PBS pH 7.4 (40 mL) mix for 5 min	– 70 ml of Freon for 5 min. – Centrifuged at 5000 × g for 10 min at 4 °C, aqueous phase collected – 30 mL PBS mixed with Freon phase for 5 min. – Centrifuged again – Aqueous phase pooled
Delicatessen foods (method 2)	30 g	NoV GI[a] HAV[a]	1. Elution 2. Concentration	2X TRIzol washing (4 ml) for 5 min	No clarification
Lettuce Hamburger	50 g	PV1[a] HAV[a]	1. Elution 2. Clarification 3. Concentration	Homogenized by blending for 2 min with 0.05 M glycine–0.14 N NaCl buffer pH 9 (350–400 mL). Hamburger samples were further processed by extraction with an equal volume of trichlorotrifluoroethane (Freon)]	1. Filtration with gauze and centrifugation 3500 × g, for 10 min

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Lettuce Strawberry	10 g lettuce 100 g strawberry	FCV[a]	1. Elution 2. Concentration	Washing with 0.05 M glycine buffer, pH 9.5, containing 3 % beef extract (100 ml) with stirring for 15 min	–
Lettuce	30 g	PV1[a]	1. Elution 2. Clarification 3. Concentration	1 M sodium bicarbonate (90 ml) with stomaching for 20 s	Centrifuged at 28,000 × g for 30 min
Green onions	9–10 g	PV1[a]	1. Elution 2. Clarification 3. Concentration	1 M sodium bicarbonate (90 ml) with pulsification for 20 s in a pulsifier	Centrifuged at 28,000 × g for 30 min
Cabbage	90 g	PV1[a]	1. Elution 2. Clarification 3. Concentration	1 M sodium bicarbonate (90 ml) with pulsification for 20 s in a pulsifier	Centrifuged at 28,000 × g for 30 min
Strawberries	100 g	PV1[a]	1. Elution 2. Clarification 3. Concentration	1 M sodium bicarbonate (15 ml) with stomaching for 1 min	Centrifuged at 28,000 × g for 3 min in presence of 1 ml CatFloc

Concentration	*Percent virus recovery*	*Notes*	*Reference*
1. Precipitation with 10 % PEG 6000–0.3 M NaCl – The mixture incubated for 2 h at 4 °C. – Centrifuged at 7000 × g for 30 min at 4 °C, – Supernatant discarded and pellet suspended in 8 ml of TRIzol **2. TRIzol clarification** – Centrifugation 8000 × g, 20 min (upper lipid layer and residual food pellet discarded) – Phenol/chloroform extraction of viral RNA	–	–	Schwab et al. (2000), Baert et al. (2008)
TRIzol clarification – Centrifugation 8000 × g, 20 min(upper lipid layer and residual food pellet discarded) – Phenol/chloroform extraction of viral RNA	–	TrIzol was used to make extraction of RNA directly during the concentration step without need for a clarification step	
1. First PEG concentration step – Supernatant adjusted to pH 7.2 to 7.3 and viruses precipitated with 6 % PEG 6000–0.3 M NaCl (overnight at 4 °C) – Centrifugation at 3500 × g for 20 min – Elution of the virus from pellet by vortex in 25 ml of 50 mM Tris-0.2 % Tween 80 (pH 9.0) **2. second PEG concentration step** – Supernatant adjusted to pH 7.2–7.3 and viruses precipitated with 12 % PEG 6000–0.3 M NaCl (for 2 h at 4 °C) – Centrifugation at 7500 × g for 15 min and resuspension in 2.5 ml of 50 mM Tris-0.2 % Tween 80 (pH 8.0).	**Lettuce:** PV1 (10–53 %) HAV (2–4 %) **Hamburger:** PV1 28–70 % HAV 2–3 %	The secondary PEG precipitation was applied to further reduce volume and to remove RT-PCR inhibitors	Leggitt and Jaykus (2000), Sair et al. (2002)

Concentration	*Percent virus recovery*	*Notes*	*Reference*
Organic flocculation – Flocculent was 3 % beef extract pH 3.5 – Centrifugation 4000 × g/15 min – Pellet dissolved in 1 mL 0.1 M phosphate buffer saline pH 9	90–100 %	–	Gulati et al. (2001)
– Ultracentrifugation at 240,000 × g for 1 h – Pellet suspended in 2 ml of M199 cell culture medium	–	–	Kurdziel et al. (2001)
– Ultracentrifugation at 240,000 × g for 1 h – Pellet suspended in 2 ml of M199 cell culture medium	–	Pulsification is better than stomaching for foods have fairly solid surface	
– Ultracentrifugation at 240,000 × g for 1 h – Pellet suspended in 2 ml of M199 cell culture medium	–	–	
– Ultracentrifugation at 240,000 × g for 1.5 h – Pellet suspended in 2 ml of M199 cell culture medium	–	Flocculation catalyst (CatFloc) used to facilitate separation of the food solids in centrifugation	

(continued)

Table 9.2 (continued)

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Raspberries	60 g	PV1[a]	1. Elution 2. Clarification 3. Concentration	1 M sodium bicarbonate (35 ml) with stomaching for 1 min	Centrifuged at 28,000 × g for 3 min in presence of 1 ml CatFloc
Fruits and vegetables (raspberry, bilberry, blackberry, currant, cherry, lettuce, radish, tomatoes)	100 g	PV1[a] HAV[a] NoV[a]	1. Elution 2. Clarification 3. Concentration	Washing with 300 mL of 100 mM Tris–HCl, 50 mM glycine, and 3 % beef extract, pH 9.5, for 20 min in a plastic bag with a filter compartment	Filtration (large plastic net) and centrifugation 10,000 × g, for 15 min at 4 °C in case of mashed raspberries the elution buffer was supplemented with 50 mM of $MgCl_2$ and 180 U of pectinase

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Frozen berries	10 g	EV[a,c] NoV[b]	1. Elution and clarification steps 2. Concentration	– Shaken with 4 mL of 0.05 M glycine-0.3 M NaCl buffer pH 9.5 for 1 min – The seeds removed by strainer – The pH adjusted to 9.5 and the mixture vortexed for more than 2 min to elute virus – 3mLof chloroform- butanol (1:1 vol/vol) was added followed by mixing for 30 s or – 90 µL of CatFloc T was added with rocking for 5 min and extraction with chloroform–butanol. or – Homogenate was centrifuged for 20 min at 12,000 × g at 4 °C. The aqueous phase was collected	
Lettuce	80 g	EV[a] NoV[a] CaCV[a]	**Method A** 1. Elution and clarification steps combined 2. Concentration	– Washing by 6 ml of PBS for 5 min – Adding equal volume of Vertrel (1,1,1,2,3,4,4,5,5,5-decafluoropentane) with shaking for 5 min – Centrifuged for 10 min at 7000 rpm and 4 °C	
			Method B 1. Elution 2. concentration (no clarification step)	– Sample was mixed for 20 min 21.3 ml (1:3, wt/vol) of beef extract (3 %, pH 9.5)	No clarification
			Method C 1. Elution-clarification combined step 2. Concentration step	– Sample was mixed with 4 ml of glycine (0.05 M)–NaCl buffer (9 g/liter, pH 9.5) and vortexed for 1 min – 3 ml of chloroform-butanol (1:1, vol/vol) and 0.5 ml of Cat-Floc were added – The mixture was centrifuged for 20 min at 12,000 x g and 4 °C	

Concentration	*Percent virus recovery*	*Notes*	*Reference*
– Ultracentrifugation at 240,000 × g for 1.5 h – Pellet suspended in 2 ml of M199 cell culture medium	–	Flocculation catalyst (CatFloc) used to facilitate separation of the food solids in centrifugation	
– Precipitation 10–50 % PEG-1.5–0.3 M NaCl (2 h—overnight at 4 °C) – Centrifugation at 10,000 × g for 30 min-2 h at 4 °C – The virus was extracted by organic extraction from the pellet by mixing with equal volumes of PBS and chloroform/butanol (1:1). Then left to stand for 5 min at 4 °C. The upper aqueous phase containing virus was recovered directly and used for analysis or after centrifugation at 6000 × g for 15 min at 4 °C, 140 µl of the aqueous phase taken for analysis	45–100 % for PV1 17 % for HAV 13 % for NLV	– Glycine and beef extract favor viral elution from acid-releasing berries. – $MgCl_2$ inhibits decrease in viral infectivity during the process. – Pectinase was added to remove residual pectin which may form hydrogel – Inhibitors and cytotoxic compounds were removed from viral concentrates by chloroform-butanol extraction	Dubois et al. (2002), Dubois et al. (2007), Baert et al. (2008), and Scherer et al. (2010)

Concentration	*Percent virus recovery*	*Notes*	*Reference*
– Precipitation with 3 mL 24 % PEG 8000–1.2 M NaCl (1 h at 4 °C). – Centrifuged for 30 min at 10,000 × g at 4 °C. The resulting pellet used for nucleic acid extraction	–	After mixing the eluent with berries, the pH had to be adjusted again to 9.5 as berries are acidic and decreased the pH below 9.5.	Le Guyader et al. (2004a), Baert et al. (2008)
– 10 % PEG-6000 + 0.3 M NaCl added to the supernatant – Incubated for 2 h at 4 °C. – Centrifuged for 30 min at 9500 rpm and 4 °C. – The pellet was suspended in 2 ml of TRIzol for RNA extraction	–	– In methods A and D RNA internal control (IC) did not amplify in PCR. It means that method A and D didn't remove PCR inhibitors. – Methods C and E were the best	Le Guyader et al. (2004b), Rutjes et al. (2006)
– Aqueous phase ultra-centrifuged for 2 h at 200,000 × g – The pellet suspended in 1 ml of sterile distilled water for nucleic acid extraction	–		
– 8 % PEG-6000 + 0.4 M NaCl was added to the supernatant and rocked for 1 h at 4 °C – Centrifuged for 30 min at 10,000 × g and 4 °C. – The pellet suspended directly in the lysis buffer of nucleic acid extraction kit	–		

(continued)

Table 9.2 (continued)

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
			Method D 1. Elution-clarification combined step 2. Concentration step	– The viruses were eluted by addition of 5 ml PBS with vortex for 5 min – Equal volume of Vertrel was added with shaking for 30 min at room temp – Centrifuged 20 min at 13,000 x g and 4 °C	
			Method E 1. Elution 2. Clarification 3. Concentration	– The viruses were eluted for 15 min by addition of 5 ml of glycine buffer (pH 8.5).	– Left for gravity settling – Supernatant was centrifuged for 20 min at 13,500 × g

Food	*Sample size*	*Virus*	*Approach*	*Elution*
Raspberries Strawberries	60–90 g	NoV[a] HAV[a]	1. Elution 2. Clarification 3. Concentration	Washed in a spinal roller for 15 min with 1 M sodium bicarbonate (100–120 ml) containing 1 % soy protein powder
Berries – Strawberry – Raspberry – Blueberry – Blackberry – Black currants **Vegetables/herbs** – Lettuce – Green onion – Mint – Parsley	15 g	HAV[a] NoV[a] RV[a]	1. Elution 2. Clarification 3. Concentration	– Viruses eluted by 60 ml of 50 mM glycine, 100 mM Tris, 1 % [wt/vol] beef extract [pH 9.5] for 15 min at room temperature with shaking
Lettuce Fruit salad Raspberries Two Ready-to-eat-dishes	50 g	NoV[a]	1. Elution 2. Clarification 3. Concentration	– Samples washed with 150 ml 0.05 M glycine-0.15MNaCl pH 9 in a stomacher bag with filter compartment on a shaking platform for 20 min
Lettuce Green onions Strawberries	2–2.5 cm^2 surface samples	HAV[a] AiV[a] FCV[a]	1. Elution	Virus eluted in 1 mL of one of the following media: 1. MEM containing 2 % FBS; pH 7.8), 2. 100 mM Tris, 50 mM glycine, 3 % beef extract, and 50 mM MgCl2; pH 9.5), 3. PBS (pH 7.2) By repeated pipetting of the demarcated area or vortexing for 2 min
			1. Elution 2. Clarification	Samples were blended in 10 mL of 0.15 M Na_2HPO_4 (pH 9.5) at high speed for 3 min

Concentration	*Percent virus recovery*	*Notes*	*Reference*
– Supernatant concentrated by ultracentifugation for 2 h at 200,000 × g – The pellet suspended in 200 µl of PBS – Nucleic acid extraction	–		
– Supernatant concentrated by a microconcentrator (Amicon, Millipore Corp., Bedford, Mass.) until volume was reduced to 200 ml	–		

Clarification	*Concentration*	*Percent virus recovery*	*Notes*	*Reference*
– Centrifuged at 8000 × g for 3 min (2 min in case of NoV) – 1 ml CatFloc + 0.5 ml pectinase were added to the supernatant – Centrifuged at 28,000 × g for 30 min	– Ultracentrifuged at 235,000 × g for 2 h (2.25 h in case of NoV) – Pellet was suspended in 500 µL of PBS	–	Pectinase was added to remove residual pectin which may form hydrogel	Rzeżutka et al. (2005), Rzeżutka et al. (2006)
– Filtered through cell strainer – The recovered elution buffer was then adjusted to pH 7.0 ± 0.2 with 9.5 M HCl – 300 µl of pectinase was added with shaking for 30 min – The extract was centrifuged at 3500 × g for 15 min	– The elution buffer centrifuged at 3500 × g for 15–45 min using centrifugal filter (100 K NMWL) – The virus eluted in 600 µl of elution buffer, pH 7.0 ± 0.2 and used for nucleic acid extraction	1.7–19.6 %	–	Butot et al. (2007)
– The filtrated part was centrifuged (10,000 × g, 15 min, 4 °C). The pH of the supernatant was adjusted to 7.2–7.4	– PEG (6 % wt/vol) and 0.3MNaCl was added then kept under shaking overnight (4 °C) – Samples were centrifuged (10,000 × g, 30 min,4 °C) – Pellets were collected for nucleic acid extraction	–	–	Baert et al. (2008)
No clarification	No concentration	67–87 % HAV 75–84 % AiV 76–88 % FCV	This method was for recovery of virus from food surface	Fino and Kniel (2008)
Centrifugation at 2300 g, and the supernatant was adjusted to pH 7.0 with 1 N HCl	No concentration	75–87 % HAV 62–70 % AiV 66–86 % FCV		

(continued)

Table 9.2 (continued)

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Tomato sauce Strawberry puree	10 g	HAV[a] PV1[a] MS2 [a] phage	1. Combined Elution-Clarification step 2. Double-step concentration	– Viruses were eluted by vortex mixed for 2 min with 105 ml of 0.05 M glycine–0.14 M NaCl buffer, pH 7.5. Clarified with Vertrel (1:2, v/v) – Centrifugation at 5000 × g for 30 min at 4 °C. (supernatant collected) – The remaining Vertrel/buffer interface was re-extracted with 105 ml of 0.5 M threonine–0.14 M NaCl, pH 7.5 (vortex for 2 min) – Centrifugation at 5000 × g for 30 min at 4 °C. (supernatant collected)	
Strawberries Lettuce	5 g 25 g	NoV[a]	1. Elution 2. Clarification 3. Double-step concentration	The virus was eluted by mixing samples with 25–250 mL of 0.05 M glycine + 100 mM Tris elution buffer (pH 9.5) for 30 min at room temp	– The elute was centrifuged at 3500 × g for 15 min after adjusting the pH to 7–7.2
Lettuce, Strawberry, Raspberries, Green onions	50 g	HAV[a] NoV[a]	1. Elution 2. Concentration by anion exchange filtration	– 50 ml of desorption buffer (0.1 M Tris–HCl (pH 7)–1 M NaCl) was added to the food samples and pipetting was carried out to desorb the viral particles	No clarification
Spinach	25 g	RV[a] HAV[a] FCV[a]	1. Elution 2. Concentration by filter adsorption plus a secondary concentration step	Samples stomached with 225 ml of washing buffer (glycine 0.05 M, NaCl 0.14 M, pH 7.5) for 90 min at room temp	No clarification

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Strawberry	25 g	MNV[a,c] FCV[a,c] NoV GI[b] RV[b] Swine HEV[b]	1. Elution 2. Clarification 3. Concentration by ultrafiltration	Viruses were eluted by shaking for 30 min with Glycine-NaCl buffer (0.05 M and 0.14 M; pH 7.5) contained 200 µl of pectinase	The elution buffer was centrifuged at 5000 × g for 5 min – The supernatant filtered through a Whatman GD/X membrane
Parsley Spinach Mix salad	10 g	HAV[a] MNV[a] NoV[a]	1. Elution 2. Clarification 3. Concentration	– Samples were homogenized with 90 mL of buffered peptone water (BPW) in a sterile plastic bag with lateral filter using pulsifier for 15 s	– The mixture was taken from the filter side to remove particulate debris, and vegetables were then rinsed with 5 mL of BPW

Concentration	*Percent virus recovery*	*Notes*	*Reference*
– Combined supernatant adjusted to pH 7.2 and mixed with 8 % (w/v) PEG-8000 and 0.3 M NaCl, – PEG mixtures were incubated overnight at 4 °C – Centrifugation at 15,000 × g for 30 min at 4 °C – Resulted pellet was resuspended in 4 ± 1 ml of PBS, supplemented 1:1 (v/v) with chloroform or Vertrel, vortex mixed for 2 min – Centrifuged at 3000 × g for 15 min at 4 °C – The aqueous phase collected for nucleic acid extraction	–	–	Love et al. (2008)
– The resulting supernatants were mixed with 8 % (wt/vol) PEG-8000 + 0.3 M NaCl and incubated for 2 h at 4 °C to precipitate viruses – The samples were centrifuged at 10,000 × g for 30 min at 4 °C, and the pellet was suspended with sterile PBS – Ultrafiltration as a secondary concentration in case of large samples (100 g)	1.9–4.8 %	–	Cheong et al. (2009)
Elutes were passed through a 24 mm nanoalumina filter (2 μm pore size, 4601 grade; Ahlstrom Filtration LLC, Mt Holly Springs, PA) at a rate of 10 ml/min The viral particles were eluted by adding 500 μl of glycine buffer (pH 9) to the filter and evacuated with air after 1 min of contact with the filter	7–32 % HAV 5–16 % NoV		Morales-Rayas et al. (2010)
1st step: – The washing buffer was passed through a Zetaplus 60 S filter for virus adsorption – Adsorbed viruses were eluted by incubating the filter at room temp. with agitation for 30 min with 10 ml of TPBG buffer (2.9 % Tryptose Phosphate Broth and 6 % glycine, pH 9.0) 2nd step: – The pH of elute was adjusted to 7.0–7.4 with HCl 1 N – Ultrafiltration in an Amicon centrifugal filtration (5000 × g, 10 min) before nucleic acid extraction	–	The method used for simultaneous recovery of bacteria and viruses from spinach	Brassard et al. (2011)

Concentration	*Percent virus recovery*	*Notes*	*Reference*
– The filtrate was concentrated by centrifugation at 5000 × g for 15 min on an ultrafiltration device (Amicon Ultra-15; Millipore, Billerica, MA)			Brassard et al. (2012)
– The resulting filtrate (95 mL) was supplemented with a final concentration of 10 % PEG 8000 and 0.3 M NaCl after gentle shaking for 1 h at 4 °C – Samples were centrifuged for 30 min at 10,000 × g at 4 °C – The pellet was immediately resuspended with 300 μL of PBS	20.7 HAV 43.5 % MNV 9.2 % NoV	The method used for simultaneous recovery of bacteria and viruses from food	Sánchez et al. (2012)

(continued)

Table 9.2 (continued)

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Lettuce Raspberry Ham	25 g	NoV[a]	1. Elution 2. Clarification 3. Concentration	– Mixing with 12.5 ml glycine buffer (pH 8.5) for 5 min	– Centrifugation of supernatant at 13,500 × g for 20 min
			1. Elution 2. Combined clarification and concentration step	– Wash with 40 ml washing buffer of the kit in a filter bag with shaking for 30 min	– Immunomagnetic separation by combining the recovered wash buffer and 100-µl magnetic beads in a 50-ml tube – Incubation with shaking at room temp. for 60 min – Removal of supernatant in magnetic stand – Washing of beads and removal of wash buffer in magnetic stand then Lysis buffer for RNA extraction
			1. Elution 2. Clarification 3. Concentration	– 50 ml of 1 M $NaHCO_3$ with 1 % soy protein – Shaking for 15 min	– Centrifugation at 6000 × g for 2 min; Supernatant mixed with 500 µl Catfloc T and 200 µl pectinase – Centrifugation at 28 000 × g for 30 min
			1. Elution 2. Clarification 3. Concentration	40 ml TGBE buffer (100 mM Tris, 50 mM glycine, 1 % beef extract, pH 9.5) in a filter bag with shaking for 20 min (keeping pH above 9)	– Centrifugation of recovered elution buffer at 10,000 × g for 15 min – pH of supernatant adjusted to 7.2. – Pectinase (300 µl), incubation at room temp. for 30 min

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Fresh fruit and vegetable salad Blueberries Cherry tomatoes	5 g	NoV[a] (different genotypes)	1. Elution 2. Clarification 3. Concentration by porcine stomach mucin-conjugated magnetic beads (PGM-MB)	– Viruses were eluted by shaking sample in a rocker at 35 rpm for 30 min at room temp with 35 mL of one of the following buffers: 1. DNase/RNase-free distilled water (ddH_2O), 2. PBS, pH 7.5), 3. Citrate-buffered saline (CBS, 0.1 M, pH3.6), 4. Glycine buffer (0.1 M glycine, 0.3 M NaCl, pH 9.5)	The eluate was clarified By: 1. Centrifugation at 10,000 × g for 15 min at 4 °C for salad, or 2. 3000 × g for 5 min for blueberries and cherry tomatoes

Concentration	*Percent virus recovery*	*Notes*	*Reference*
– Ultrafiltration by centrifugation of supernatant with a micro-concentrator device – 500–1000 µl of concentrate for RNA extraction	1–20 % lettuce 2–5 % ham 0.1–1 % raspberry	Commercial microconcentrator tube Amicon Ultra-15 Centrifugal Filter Device (Millipore, USA)	Summa et al. (2012)
	3 % lettuce 1–5 % ham 0.4–4 % raspberry	Commercial NoroCheck IMS kit (Kim Laboratories, USA)	
– Ultracentrifugation of supernatant at 50,000 × g for 3 h 20 min – Resuspension of pellet in 500 µl of PBS for RNA extraction	9–19 % lettuce 9–70 % ham 0.8–4 % raspberry	Yielded the highest recovery efficiencies in lettuce and ham	
– PEG8000 precipitation, incubation at 4 °C for 2 h – Centrifugation at 10,000 × g for 30 min; pellet was resuspened in 500 µl of PBS – Treatment with chloroform-butanol mixture 500 µl then incubation 5 min – Centrifugation at 10,000 × g for 15 min – Aqueous phase for RNA extraction	5–19 % lettuce 7–47 % ham 4–28 % raspberry	Commercial A BagFilter® F (Interscience, USA) Dilution of RNA extract increased the recovery because of dilution of inhibitors	

Concentration	*Percent virus recovery*	*Notes*	*Reference*
– 35 mL of clarified eluate +15 mL citrate-buffered saline (CBS, 0.1 M, pH 3.6) +100 µL of PGM-MB suspension were mixed on a Roto-Shake Genie shaker for 30 min. – Separation of beads by a 50-ml magnetic separation rack for 30 min then collected beads were washed three times by PBS then resuspended in 140 µL of PBS for RNA extraction.	6–30 %	–	Pan et al. (2012)

(continued)

Table 9.2 (continued)

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Cabbage Lettuce Ham	10 g	FCV[a,c] NoV[a]	1. Elution 2. Clarification 3. Concentration	Soaking with 40 ml elution buffer in a stomacher bag Shaking 120 rpm for 10 min **Buffers:** 1. PBS consisting of 138 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.2 mM KH_2PO_4, pH 7.4 2. Tris-glycine buffer (100 mM Tris, 50 mM glycine, pH 9.5)	– Filtration in a polyester mesh (100 mesh) to remove food debris – Centrifugation at 1880 × g for 30 min
Lettuce	25 g	NoV[a] MNV[a]	1. Elution 2. Clarification 3. Double-step concentration	– The sample stomached for 2 min with 225 mL of the elution buffer in a plastic filter bag (Nasco®, Fort Atkinson, Wisconsin, USA) Tested buffers: 1. PBS pH 7.2, 2. Glycine 0.05 M/NaCl 0.15 M pH 9.5 3. Glycine 0.05 M/Tris–HCl 0.1 M pH 9.5	The supernatant was clarified by centrifugation at 5000 × g for 15 min at 4 °C

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Sesame Leaves Carrots, Onions Radish	10–25 g	PV1[a] NoV[a]	1. Elution 2. Clarification	Samples shaken for 3 h with 150 mL elution buffer at room temp. Buffers: – 0.25 M threonine / 0.3 M NaCl (pH 9.5) – 0.25 M glycine / 0.14 M NaCl (pH 9.5)	– Filtering with a syringe filter (0.45 μm) porosity
Raspberry	25 g	MNV[a] ECBO[a] MS2[a]	1. Elution 2. Clarification 3. Double-step concentration	Elution by adding 40 mL of TGBE buffer (Tris–HCl 100 mM, glycine 50 mM, 1 % beef extract, pH 9.5) and 30 units of pectinase to the sample in a mash filter bag with shaking incubation (400 rpm, 20 min, at room temperature) and keeping the pH 9.5. ECBO virus is added	– The eluate was clarified by centrifugation (10,000 × g, 30 min, at 4 °C)

Concentration	*Percent virus recovery*	*Notes*	*Reference*
– Addition of ascorbic acid – Addition of 0.3 g of amorphous calcium phosphate (ACP) particles Vigorous stirring for 1 h at room temp – Centrifugation at 1880 × g for 10 min – Dissolution of ACP particles by addition of 3.3 M citric acid RNA extraction using QIAamp Viral RNA MiniKit	FCV 32–33 %, 50–55 %, and 37–46 % NoV 12–41 %, 30–57 %, and 20–26 % for cabbage, lettuce, and ham, respectively	– Ascorbic acid was added to improve viral recovery based on previous results – Tris-glycine buffer was the best	Shinohara et al. (2013)
– Removal of cations from the filter by acid rinse (H_2SO_4, 5 mM, pH 3.0) – samples were concentrated by filtration on 0.45 µm negatively charged membrane Stericup filter (250 ml) (Nihon, Millipore, USA) – The final concentration of 25 mM of $MgCl_2$ was adjusted in the solution obtained from the recovery stage, and the pH was adjusted to ≤5.0 – Ultrafiltration step using a Centriprep Concentrator® 50 (Nihon, Millipore), to obtain concentrate at a final volume of 2 ml	5.2–9.8 % for PBS 0.2–18 % for glycine-NaCl 10.8–33.3 % for glycine-tris	–	Corrêa and Miagostovich (2013)

Concentration	*Percent virus recovery*	*Notes*	*Reference*
No concentration	25–90 % Depends on the elution buffer type	Recovery from leafy samples was more efficient than from root vegetables	Moon et al. (2013)
– Adjusting the supernatant pH immediately to 7.0 ± 0.2. – Mixing with 10 % (w/v) PEG- 6000 (5×) and 300 mM NaCl, and then incubation for 1 h at 4 °C on a shaking platform (400 rpm). – Centrifugation (10,000 × g, 30 min, at 4 °C) – Pellet's suspended in 500 µL of PBS pH 7.2. – Mixing with 500 µL of chloroform: butanol, 1:1 (v/v). After 5 min at room temp – Centrifugation (10,000 × g, 15 min, at 4 °C). The upper aqueous phase was recovered	MNV 20.3 %	This method is the IOS technical specifications document, a method for virus detection and quantification in food, including raspberries	ISO/TS 15216-1 (2013), ISO/TS 15216-2 (2013)

(continued)

Table 9.2 (continued)

Food	*Sample size*	*Virus*	*Approach*	*Elution*	*Clarification*
Raspberry	25 g	MNV[a] ECBO[a,c] MS2[a,c]	1. Direct RNA extraction 2. Homogenate clarification 3. RNA purification	**1. Immersion in lysis buffer** The sample was placed with 3 mL of guanidium isothiocyanate 5 (NucliSENS®easyMAGTM Lysis Buffer, 280134) and ECBO virus (100 µL) in glass tubes for 5 min and maintained in continuous rotation. **2. RNA clarification** The homogenate was clarified by centrifugation (15,000 × g, 5 min, at room temperature) **3. RNA Purification** – The entire clarified supernatant was processed for RNA purification using NucliSENS®easyMAG™platform (bioMérieux) with the "off-board specific protocol" according to the manufacturer's instructions	

Food	*Sample size*	*Virus*	*Approach*	*Elution*
Strawberry	25 g	NoV[a] MNV[a]	1. Elution 2. Concentration by skim milk flocculation method	Elution by two different elution buffers: 1. PBS 1x (pH7.2) 2. Glycine Tris–HCl (pH 9.5) 400 mL for 1 h washing in glass beaker or 225 mL of buffer for 30 min if using filter bags (Nasco®, Fort Atkinson, Wisconsin, USA)

AiV aichi virus, *FCV* feline calicivirus, *HEV* hepatitis E virus, *NoV* human norovirus, *PV1* poliovirus type 1, *RV* human rotavirus, *EV* enterovirus, *MNV* murine norovirus, *CaCV* canine calicivirus, *ECBO* enteric cytopathogenic bovine orphan virus, *MS2* male specific coliphage, *PEG* polyethylene glycol

[a]Seeded and recovered virus

[b]Detected in an outbreak-implicated sample

[c]Extraction control (internal process control)

Concentration	*Percent virus recovery*	*Notes*	*Reference*
	MNV 46.2 %	Direct RNA extraction proved to be more effective (best recovery rate), faster (<8 h) and simpler (fewer steps) than the one proposed in the CEN ISO standard method when it came to detecting enteric viruses on raspberries	Perrin et al. (2015)

Clarification	*Concentration*	*Percent virus recovery*	*Notes*	*Reference*
No clarification	– Adjusting the pH of eluate to 3.5 using HCl 6 N – Adding skimmed milk at a final concentration of 1 % with constant stirring for 3 h – Centrifugation (8000 × g for 30 min) — Re-suspension of the pellets in 1 mL of a phosphate buffer (pH 7.5; Na2HPO4 0.2 M/NaH2PO4 0.2 M; 1:2 v/v)	–	Recovery percentage of 2.5 and 32 times higher than PEG precipitation and filtration methodologies	Melgaço et al. (2016)

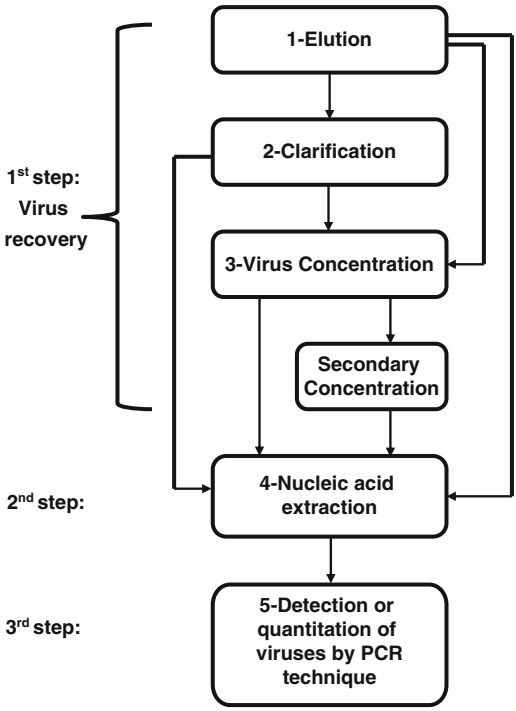

Figure 9.1 The most frequently used strategies for virus detection in foods; the first step (virus recovery) consists of three sub-steps leading to virus recovery from food.

(Le Guyader and Atmar 2007). This chapter focuses only on recovery of viral pathogens from fruits, vegetables and minimal processed foods. The methods used with shellfish samples are not included here as they are addressed in another chapter.

Recently, a few standard methods have been posted by standardization agencies for the detection of specific viral pathogens in certain types of foods. In 2013, the International Organization for Standardization (ISO) published a technical specifications document of a horizontal method for detection of HAV and NoV in foods using real-time RT-PCR (ISO/TS 15216-1:2013; ISO/TS 15216-2:2013). In January 2014, the U.S. Food and Drug Organization (US-FDA 2015) posted in its Bacteriological Analytical Manual (BAM), the most recent standard method for HAV detection in foods. Based on a survey of the published literature since 1982, the various protocols for virus detection in foods (except shellfish) utilize two main approaches: (1) Recovery of the viral particles approach and (2) Direct RNA recovery approach, which skips the elution, clarification and concentration steps.

3.1. The Approach of Viral Particle Recovery

Most of the methods under this approach are based on elution of virus particles from the food surface using an appropriate buffer followed by clarification and/or concentration of the eluted viruses. Most of the virus recovery approaches

follow a three-step protocol (Table 9.2, Fig. 9.1) consisting of: (1) elution of virus particles from the food matrix, (2) clarification of the virus-containing eluate, and (3) concentration of the recovered virus particles in a small final sample volume (Cliver et al. 1983; Schwab et al. 2000; Leggitt and Jaykus 2000; Sair et al. 2002; Kurdziel et al. 2001; Dubois et al. 2002; Dubois et al. 2007; Scherer et al. 2010; Rzeżutka et al. 2005; Rzeżutka et al. 2006; Butot et al. 2007; Baert et al. 2008; Cheong et al. 2009). Some of the protocols add a secondary concentration step to obtain the virus particles in a more concentrated and purified form in order to increase the chances of detection by RT-PCR (Ward et al. 1982; Love et al. 2008; Brassard et al. 2011; Corrêa and Miagostovich 2013).

Some other approaches follow a two-step protocol in which the clarification step is combined with the elution step followed by a concentration step (Le Guyader et al. 2004a; Baert et al. 2008; Le Guyader et al. 2004b; Rutjes et al. 2006; Love et al. 2008). Some investigators do not perform the clarification step; they follow a concentration step directly after the elution step (Schwab et al. 2000; Baert et al. 2008; Gulati et al. 2001; Le Guyader et al. 2004b; Rutjes et al. 2006). In one protocol, RNA extraction was performed directly after the elution step in a one-step protocol (Fino and Kniel 2008). Some of the above mentioned steps are summarized in the following subsections.

3.1.1 Elution of Viral Particles from Food Matrices

This step appears to be inspired from a method used to recover bacteriophage and poliovirus from water by adsorption-elution on a chromatographic column of activated carbon (Cookson and North 1967; Gerba et al. 1975), which is referred to as, "acidic adsorption-elution technique". This extraction technique involves the adsorption of viral particles to the negatively charged activated carboxyl groups by addition of acidic buffer and changing the ionic strength, which makes the positively charged-amino groups on the virus become associated with the negatively charged carboxyl groups on the carbon by electrostatic attraction. Then by adding a basic pH buffer with different ionic strength, the virus can be released due to the change of its charge in an alkaline pH environment. The same principle was used later to recover enteroviruses from oyster tissues by acidic adsorption (Sobsey et al. 1975) followed by a next step (elution) in which the supernatant is discarded and the viruses are eluted from the oyster tissue pellet using either a neutral or basic solution of glycine-PBS.

The acidic adsorption-elution technique is used for extraction of foodborne viruses from a broad range of carbohydrate/water-based foods, fatty/protein-based foods as well as shellfish. An overview of the elution buffers is shown in Table 9.3. Generally, the elution step is similar in most protocols. Most of them use alkaline buffer with a pH ranging from 8 to 10.5 to elute the viral particles from the surface of fruits and vegetables. The alkaline pH detaches the viruses by breaking the electrostatic and hydrophobic interactions between fruit or vegetable surfaces and viruses (Croci et al. 2008). On the other hand, the acidic medium encourages the viral particles' binding to food

Table 9.3 Buffers used for elution of viral particles from foods

Elution buffer	*pH*	*Food material*	*Sample/Buffer ratio (w/v)*	*Procedure/Time*	*References*
PBS	9.0	Lettuce, cabbage, celery, spinach	1:24	Shaking/7–10 min	Ward et al. (1982)
PBS	7.4	Delicatessen foods	1:4	Mixing/5 min	Schwab et al. (2000)
PBS	7.2	Lettuce, green onions, strawberries	2:1	Blending/3 min	Fino and Kniel (2008)
PBS (0.15 M)	9.5	Lettuce, green onions, strawberries	1:5	Blending/3 min	Fino and Kniel (2008)
PBS	7.5	Fresh fruit and vegetable salad	1:7	Shaking/30 min	Pan et al. (2012)
PBS	7.2	Lettuce	1:5	Stomaching/2 min	Corrêa and Miagostovich (2013)
PBS	7.2	Strawberry	1:16	Shaking/30 min	Melgaço et al. (2016)
PBS (8.1 mM)	7.4	Cabbage, lettuce, ham	1:4	Shaking/30 min	Shinohara et al. (2013)
Sodium bicarbonate 1 M	–	Lettuce	1:3	Stomaching/20 s	Kurdziel et al. (2001)
Sodium bicarbonate 1 M	–	Green onions, cabbage	1:9 or 1:1	Pulsification/20 s	Kurdziel et al. (2001)
Sodium bicarbonate 1 M	–	Strawberries	5:1	Stomaching/20 s	Kurdziel et al. (2001)
Sodium bicarbonate 1 M	–	Raspberries	2:1	Stomaching/20 s	Kurdziel et al. (2001)
Sodium bicarbonate 1 M + 1 % soy protein powder	–	Raspberries, strawberries	1:2	Spinal roller/1 min	Rzeżutka et al. (2005), Rzeżutka et al. (2006)
Sodium bicarbonate 1 M + 1 % soy protein powder	–	Lettuce, raspberry, ham	1:2	Shaking/15 min	Summa et al. (2012)
Glycine–NaOH buffer	8.8	Lettuce	1:4	Homogenization/5 min	Cliver et al. (1983)
Glycine–NaOH buffer	10.8	Strawberry	1:4	Homogenization/5 min	Cliver et al. (1983)
Glycine 0.05 M–NaCl 0.14 N	9.0	Lettuce, hamburger	1:8	Blending/2 min	Leggitt and Jaykus (2000)
Glycine 0.05 M–NaCl 0.3 M	9.5	Berries	4:10	Shaking/1 min	Le Guyader et al. (2004a)
Glycine 0.05 M–NaCl	9.5	Berries	1:20	Vortexing/1 min	Le Guyader et al. (2004a)
Glycine 0.05 M–NaCl 0.15 M	9.0	Lettuce, fruit salad, raspberries	1:3	Stomaching/20 min	Baert et al. (2008)
Glycine 0.05 M–NaCl 0.14 M	7.5	Tomato sauce, strawberry puree	1:10	Vortexing/2 min	Love et al. (2008)
Glycine 0.05 M–NaCl 0.14 M	7.5	Spinach	1:12	Stomaching/90 min	Brassard et al. (2011)
Glycine 0.05 M–NaCl 0.14 M	7.5	Strawberry		Shaking/30 min	Brassard et al. (2012)

Glycine 0.05 M- NaCl 0.15 M	9.5	Lettuce	1:5	Stomaching/2 min	Corrêa and Miagostovich (2013)
Glycine 0.25 M–NaCl 0.14 M	9.5	Sesame leaves, carrots, onions, radish	1:6 to 1:10	Shaking/3 h	Moon et al. (2013)
Glycine 0.1 M–NaCl 0.3 M	9.5	Fresh fruit and vegetable salad	1:7	Shaking/30 min	Pan et al. (2012)
0.1 M Tris–HCl–1 M NaCl	7.0	Lettuce, strawberry, raspberries, green onions	1:1	Pipetting—dispersing many times	Morales-Rayas et al. (2010)
Glycine 0.05 M–Tris–HCl 100 mM	9.5	Strawberry, lettuce	1:10	Shaking /30 min	Cheong et al. (2009)
Glycine 0.05 M–Tris–HCl 0.1 M	9.5	Lettuce	1:5	Stomaching/2 min	Corrêa and Miagostovich (2013)
Glycine–Tris–HCl	9.5	Strawberry	1:16	Shaking/30 min	Melgaço et al. (2016)
Glycine–Tris–HCl	8.5	Lettuce, raspberry, ham	1:5	Mixing/5 min	Summa et al. (2012)
Glycine 50 mM–Tris 100 mM +1 % beef extract	9.5	Lettuce, raspberry, ham	1:2	Shaking/20 min	Summa et al. (2012)
Glycine 50 mM–Tris 100 mM +1 % beef extract	9.5	Berries, vegetables, herbs	1:6	Shaking/15 min	Butot et al. (2007)
Glycine 50 mM–Tris 100 mM +3 % beef extract +50 mM $MgCl_2$	9.5	Lettuce, green onions, strawberry	2:1	Repeated pipetting of the demarcated area/2 min	Fino and Kniel (2008)
Glycine 50 mM–Tris 100 mM +3 % beef extract	9.5	Lettuce, berries, radish, tomatoes	1:3	Washing in plastic bag/20 min	Dubois et al. (2002)
Glycine 0.05 M +3 % beef extract	9.5	Lettuce, strawberry	1:10 or 1:1	Stirring/15 min	Gulati et al. (2001)
Beef extract 3 %–NaOH buffer	8.0	Lettuce, celery, carrot, radish	1:2	Mixing /60 min	Badawy et al. (1985)
Threonine 0.25 M–NaCl 0.3 M	9.5	Sesame leaves, carrots, onions, radish	1:6 to 1:10	Shaking/3 h	Moon et al. (2013)
Buffered peptone water	–	Parsley, spinach, mix salad	1:9	Pulsification/15 s	Sánchez et al. (2012)
Citrate-buffered saline 0.1 M	3.6	Fresh fruit and vegetable salad	1:7	Shaking/30 min	Pan et al. (2012)
DNase/RNase-free distilled water	–	Fresh fruit and vegetable salad	1:7	Shaking/30 min	Pan et al. (2012)
MEM containing 2 % FBS	7.8	Lettuce, green onions, strawberries	2:1	Stomaching/20 min	Fino and Kniel (2008)

surface. The latter hinders the elution of the virus and reduces detection sensitivity (Dubois et al. 2002). For example, for acidic fruits such as raspberries and strawberries, the elution buffers must be more basic than the elution buffers used with neutral foods such as lettuce. This is because the acidic pH of the food can reduce the pH of the eluent solution, which may decrease the pH below neutrality with a reduction in the elution capacity and consequent reduction in detection sensitivity (Croci et al. 2008; Stals et al. 2012). Therefore the food products (particularly fruits) which contain acidic substances, an alkaline Tris-based buffer is usually employed (Dubois et al. 2002; Dubois et al. 2007; Baert et al. 2008; Scherer et al. 2010; Summa et al. 2012). The presence of "Tris" in the elution buffer provides an appropriate medium when the eluate is to be used in RT-PCR. On the other hand, Tris may decrease viral infectivity (Croci et al. 2008). To overcome the viral infectivity loss caused by Tris-containing eluents, the eluents are often supplemented with $MgCl_2$ as previous studies have reported that enteric viruses (Wallis and Melnick 1962; Parry and Mortimer 1984) show more resistant to inactivation in the presence of $MgCl_2$ (Dubois et al. 2002).

In addition to alkaline buffers, acidic or neutral elution buffers have also been used: (1) distilled water for elution of rotavirus from lettuce, carrot, radish, and celery (Badawy et al. 1985) and NoV from fruit and vegetable salad (Pan et al. 2012); (2) phosphate buffer (pH 7.2–7.6) for elution of viruses from lettuce, fresh strawberries and other vegetables and fruits (Bidawid et al. 2000; Schwab et al. 2000; Fino and Kniel 2008; Shinohara et al. 2013; Melgaço et al. 2016); (3) neutral Tris–HCl buffer for extraction of HAV and NoV from lettuce, strawberries, raspberries, and green onions (Morales-Rayas et al. 2010); (4) acidic citrate buffer saline (pH 3.6) for virus recovery from fresh fruits and vegetable salad (Pan et al. 2012); (5) sodium bicarbonate alone (Kurdziel et al. 2001) or with 1 % soya protein in sodium bicarbonate buffer (Rzeżutka et al. 2005; Rzeżutka et al. 2006; Summa et al. 2012) for elution of enteric viruses from soft fruit and salad; (6) beef extract-NaOH buffer (pH 8) for elution of viruses from fresh produce such as lettuce, celery, carrot and radish (Badawy et al. 1985). Some bacteriological or cell culture media e.g., buffered peptone water (Sánchez et al. 2012) and minimal essential medium containing fetal bovine serum (Fino and Kniel 2008), have also been used for virus elution.

In a majority of protocols, the elution buffers have been used in combination with specific supplements to play a certain role in enhancing the recovery of viral particles from foods. Beef extract and glycine in addition to soya protein are the most frequently used supplements used with elution buffers because they are able to reduce non-specific virus adsorption to the food matrix during the elution step (Dubois et al. 2002). The principle of supplementing the elution buffer with beef extract or glycine was inspired from the use of protein supplements to facilitate the recovery of viruses from filter membranes that were used for recovering enteroviruses from water (Wallis et al. 1972). Proteins, amino acids and beef extract adsorbs to the membrane (or food matrix), exchanging them for virus and thereby eluting virus from

the membrane or food surface (Wallis et al. 1972; Metcalf et al. 1980). Furthermore, the high protein content of beef extract facilitates flocculation of viruses on PEG molecules during the next concentration step (Dubois et al. 2002; Stals et al. 2012).

The contact between the sample and the elution buffers is not enough for the viral particle to be released into the elution buffer. The viruses need the assistance of mechanical force to be washed out of the food sample and into the elution buffer. Also it is important to decrease the needed time of elution and enhance its efficiency. Many methods have been used for mechanically assisting virus elution e.g., repeating pipetting (Fino and Kniel 2008; Morales-Rayas et al. 2010), shaking (Ward et al. 1982; Dubois et al. 2002; Le Guyader et al. 2004a; Pan et al. 2012; Shinohara et al. 2013; Melgaço et al. 2016), blending (Fino and Kniel 2008), stomaching (Kurdziel et al. 2001; Baert et al. 2008; Brassard et al. 2011; Corrêa and Miagostovich 2013), pulsifying (Kurdziel et al. 2001; Sánchez et al. 2012), stirring (Gulati et al. 2001), and vortexing (Le Guyader et al. 2004a; Love et al. 2008).

The selection of one of the mentioned methods depends on the available facilities in the laboratory, the characteristics of the food being tested, and the expected location of the virus in the food (on surface or internal). Regarding food characteristics, Kurdziel et al. (2001) preferred to use the pulsifier for 15 s in case of green onion and cabbage instead of stomaching because onion and cabbage are more rugged than berries. Gentle mechanical forces such as rinsing by hand, shaking in a sterile tube or plastic bag, or vortexing are most often used to elute viruses from the outer surface of foods, especially for foods with smooth outer surfaces like leafy vegetables (lettuce, spinach). For foods with rough outer surfaces and when expecting internal contamination, the use of more intensive mechanical forces such as stomaching, pulsifying, and blending are recommended. With regard to the operational time of applying the mechanical force, there is no clear criterion. Generally, the operation times range from few seconds, in case of pulsification (Kurdziel et al. 2001), to 90 min of stomaching (Brassard et al. 2011). The other types of mechanical forces fall within this range.

3.1.2 Clarification of the Virus Eluate

The virus elution step results in an alkaline eluate solution that contains viruses along with food impurities and other constituents with a size much larger than that of virus particles. The retention of these impurities in the viral eluates may lead to: (1) hindering of the next concentration step by clogging the filter membranes used in the concentration step, (2) decreasing the efficiency of the viral RNA extraction step, and (3) inhibiting the PCR reaction due to the presence of PCR inhibitors. Hence, it is very important to clarify the virus eluate before starting the concentration and/or virus detection steps.

Several clarification methods (Table 9.4) have been applied with various types of food samples or eluates. In general, the clarification step must be done

Table 9.4 Methods used for clarification of virus-containing eluates

Clarification technique	*Pretreatment*	*Supplement*[a]	*Reference*
Filtration on glass wool	Eagle's basal maintenance medium +2 % fetal calf serum	–	Ward et al. (1982)
Filtration (Whatman paper or cellulose triacetate)	–	CatFloc	Cliver et al. (1983)
Filtration (0.2 μm filter)	pH adjusted to pH 7.0 ± 0.2	–	Badawy et al. (1985)
Filtration (lateral filter of the extraction plastic bag)	–	–	Sánchez et al. (2012)
Filtration (syringe filter of 0.45 μm porosity)	–	–	Moon et al. (2013)
Filtration with (cheesecloth) then centrifugation 3500 × g/10 min	–	Freon	Leggitt and Jaykus (2000)
Filtration (large plastic net) then centrifugation 10,000 × g/15 min at 4 °C	–	$MgCl_2$ + Pectinase	Dubois et al. (2002), Dubois et al. (2007), and Baert et al. (2008)
Filtration through cell strainer then centrifugation at 3500 × g/15 min	pH adjusted to 7.0 ± 0.2	Pectinase	Butot et al. (2007)
Filtration in a polyester (100 mesh) then centrifugation at 1880 × g/30 min	–	–	Shinohara et al. (2013)
Centrifugation at 5000 × g/5 min then filtration of the supernatant through a Whatman GD/X membrane	–	–	Brassard et al. (2012)
Centrifugation	–	$MgCl_2$ + bentonite	Cliver et al. (1983)
Centrifugation at 28,000 × g for 30 min	–	CatFloc	Kurdziel et al. 2001
Centrifugation for 20 min at 12,000 × g at 4 °C. Collection of aqueous phase	–	Chloroform-butanol + CatFloc	Le Guyader et al. (2004a), Baert et al. (2008)

Centrifugation 10 min at 7000 rpm and 4 °C	–	Vertrel RX	Le Guyader et al. (2004b), Rutjes et al. (2006)
Centrifugation for 20 min at 12,000 × g and 4 °C	–	Chloroform-butanol + CatFloc or Vertrel	Le Guyader et al. (2004b), Rutjes et al. (2006)
Centrifugation at 13,500 × g/20 min	Gravity settling	–	Le Guyader et al. (2004b), Rutjes et al. (2006)
Centrifugation at 28,000 × g/30 min	–	CatFloc + Pectinase	Rzeżutka et al. (2005), Rzeżutka et al. (2006)
Centrifugation at 10,000 × g/15 min, 4 °C	–	–	Baert et al. (2008)
Centrifugation at 2300 × g	–	–	Fino and Kniel (2008)
Centrifugation at 5000 × g/30 min at 4 °C	–	Vertrel RX	Love et al. (2008)
Centrifugation at 3500 × g/15 min	pH adjusted to 7.0 ± 0.2	–	Cheong et al. (2009)
Centrifugation at 10,000 × g/15 min at 4 °C or 3000 × g/5 min	–	–	Pan et al. (2012)
Centrifugation at 5000 × g/15 min at 4 °C	–	–	Corrêa and Miagostovich (2013)
Centrifugation at 10,000 × g/30 min, at 4 °C	–	–	ISO/TS 15216-1 (2013), ISO/TS 15216-1-2 (2013)
Two times centrifugation at 5000 × g/10 min at 4 °C	–	Freon	Schwab et al. (2000), Baert et al. (2008)
Two times centrifugation—6000 × g for 2 min and 28 000 × g/30 min	–	CatFloc + Pectinase	Summa et al. (2012)

CatFolc cationic flocculent

[a]Vertrel: 1,1,1,2,3,4,4,5,5,5-decafluoropentane

under alkaline conditions (high pH) to prevent the re-adsorption of viruses to the fruit or vegetable matter. The most commonly used method for clarification of viral eluate is the use of a single low speed centrifugation step (Table 9.4) which is enough for separating the fruit and vegetable matter in a pellet while viruses remain suspended in the supernatant (Cliver et al. 1983; Kurdziel et al. 2001; Le Guyader et al. 2004a; Le Guyader et al. 2004b; Rzeżutka et al. 2005; Rutjes et al. 2006; Rzeżutka et al. 2006; Baert et al. 2008; Fino and Kniel 2008; Love et al. 2008; Cheong et al. 2009; Pan et al. 2012; Corrêa and Miagostovich 2013; ISO/TS 15216-1:2013; ISO/TS 15216-2:2013). Also a two-step centrifugation method has been used for obtaining a more pure eluate (Schwab et al. 2000; Baert et al. 2008; Summa et al. 2012). The centrifugation speeds range from 3500 to 28,000 × g for 2–30 min at room temperature or at 4 °C.

The second main method for clarification of virus eluate is filtration through different kinds of filters such as glass wool (Ward et al. 1982), Whatman or cellulose triacetate filters (Cliver et al. 1983), syringe filters of 0.2–0.45 μm porosity (Badawy et al. 1985; Moon et al. 2013), and the filter compartment of certain plastic homogenization bags (Baert et al. 2008; Sánchez et al. 2012). In some protocols, a two-step clarification method is used, which consists of filtration and centrifugation (Leggitt and Jaykus 2000; Dubois et al. 2002; Butot et al. 2007; Dubois et al. 2007; Baert et al. 2008; Shinohara et al. 2013; Brassard et al. 2012).

Many clarification protocols perform a specific pretreatment of the filters and/or add important supplements to the viral eluates (see Table 9.4) to increase the efficiency of the clarification step. For example, glass wool filters were pretreated with Eagle's basal maintenance medium plus 2 % fetal calf serum to prevent loss of virus by adsorption to the filter itself (Ward et al. 1982). In other studies, after obtaining the alkaline viral eluate, the pH was adjusted to 7.0 ± 0.2 prior to filtration for preventing the adsorption of virus particles to the filter itself (Badawy et al. 1985; Butot et al. 2007; Cheong et al. 2009). Cat-Floc™ (polydimethyldiallyl ammonium chloride, MW 500,000) is a cationic or polyelectrolyte flocculent produced by the Calgon Corp., Pittsburgh, PA. It was added as a supplement to the viral eluate to facilitate separation of the food solids during centrifugation by improving flocculation (Cliver et al. 1983; Kurdziel et al. 2001; Le Guyader et al. 2004a; Le Guyader et al. 2004b; Rzeżutka et al. 2005; Summa et al. 2012). Its use as a supplement for eluate clarification was inspired from its use as an aid for recovery of enteroviruses from oysters (Kostenbader and Cliver 1972). Bentonite is another flocculent used for enhancing the separation of the remaining food matter during centrifugation (Cliver et al. 1983). Before centrifugation, Freon (1, 1, 2-trichloro-1, 2, 2-trifluoroethane) is added to the virus eluate as an organic solvent to remove PCR inhibitors (such as lipids) (Leggitt and Jaykus 2000; Schwab et al. 2000; Baert et al. 2008). Freon is able to extract lipids and lipid bilayers without extracting proteinaceous (polar) material such as non-enveloped viruses (Stals et al. 2012). However, use of Freon has been progressively eliminated because of its implications in ozone layer depletion and accompanying environmental

concerns. Vertrel® XF(1,1,1,2,3,4,4,5,5,5-decafluoropentane) is a newly developed environmentally friendly Freon substitute (Le Guyader et al. 2004b; Rutjes et al. 2006; Love et al. 2008). A mixed organic solvent (chloroform-butanol; 1:1 v/v) is often added to the virus eluate in a number of protocols also to remove PCR inhibitory and cytotoxic substances (Le Guyader et al. 2004a; Le Guyader et al. 2004b; Rutjes et al. 2006; Baert et al. 2008; Love et al. 2008). Pectinase can be added to remove residual pectin during clarification to prevent jelly formation during neutralization of the eluate (Dubois et al. 2002; Dubois et al. 2007; Baert et al. 2008; Rzeżutka et al. 2005; Rzeżutka et al. 2006; Butot et al. 2007; Summa et al. 2012). Without the addition of Pectinase, jellies can form in eluates containing juices from soft fruits such as raspberries and frozen fruits. In some protocols, magnesium chloride is added to the elution buffer to minimize a reduction in viral infectivity during the elution and clarification processes (Cliver et al. 1983; Dubois et al. 2002; Dubois et al. 2007; Baert et al. 2008).

3.1.3 Concentration Step

This is one of the most important steps in any protocol of virus recovery from foods. Its importance is derived from the fact that in contaminated foods, the virus concentration is very low. To avoid false negative results, it is necessary to concentrate the eluted virus in a small sample volume so that it can be easily assayed by molecular or conventional methods. Several methods of concentrating the virus eluate are available (Tables 9.2 and 9.5), which continuously are being updated and enhanced in an effort to improve virus recovery and remove PCR inhibitors. These methods employ various techniques e.g., organic flocculation (Gulati et al. 2001; Melgaço et al. 2016), polyethylene glycol (PEG) precipitation (Cliver et al. 1983; Schwab et al. 2000; Dubois et al. 2002; Le Guyader et al. 2004a,b; Rutjes et al. 2006; Dubois et al. 2007; Baert et al. 2008; Love et al. 2008; Cheong et al. 2009; Scherer et al. 2010; Sánchez et al. 2012; Summa et al. 2012; ISO/TS 15216-1:2013; ISO/TS 15216-2:2013), ultracentrifugation (Kurdziel et al. 2001; Le Guyader et al. 2004b; Rzeżutka et al. 2005; Rutjes et al. 2006; Rzeżutka et al. 2006; Butot et al. 2007), ultrafiltration (Cliver et al. 1983; Le Guyader et al. 2004b; Rutjes et al. 2006; Summa et al. 2012; Brassard et al. 2012), filter adsorption-elution (Ward et al. 1982; Brassard et al. 2011; Corrêa and Miagostovich 2013), adsorption-elution on amorphous calcium phosphate (ACP) particles (Shinohara et al. 2013), and separation using magnetic beads coated with virus-specific ligands (Summa et al. 2012; Pan et al. 2012). A summary of these concentration methods follows:

Organic Flocculation This method is based on the adsorption of eluted, positively charged virus particles on protein flocculate under acidic conditions. The pH of the virus eluate is adjusted to pH 3.5 prior to adding the flocculent. The flocculate with adsorbed virus is separated by centrifugation and the pel-

Table 9.5 Concentration methods for viruses eluted from foods

Concentration method	*Secondary concentration step*	*Reference*
Organic flocculation by 3 % beef extract	–	Gulati et al. (2001)
Organic flocculation by 1 % skim milk	–	Melgaço et al. (2016)
PEG precipitation (30 % PEG20,000)	Ultrafiltration 275,000 × g/2 h	Cliver et al. (1983)
PEG precipitation (10 % PEG6000+ 0.3 M NaCl)	2nd PEG precipitation (12 % PEG 6000 + 0.3 M NaCl)	Schwab et al. (2000), Baert et al. (2008)
PEG precipitation (10 % PEG6000+ 1.5–0.3 M NaCl)	Chloroform/butanol	Dubois et al. (2002), Dubois et al. (2007), Baert et al. (2008), Scherer et al. (2010)
PEG precipitation (24 % PEG8000+ 1.2 M NaCl)	–	Le Guyader et al. (2004a), Baert et al. (2008)
PEG precipitation (10 % PEG6000+ 0.3 M NaCl)	–	Le Guyader et al. 2004b; Rutjes et al. 2006
PEG precipitation (8 % PEG6000+ 0.4 M NaCl)	–	Le Guyader et al. (2004b), Rutjes et al. (2006)
PEG precipitation (6 % PEG6000+ 0.3 M NaCl	–	Baert et al. (2008)
PEG precipitation (8 % PEG8000+ 0.3 M NaCl)	Chloroform or Vertrel	Love et al. (2008)
PEG precipitation (8 % PEG8000+ 0.3 M NaCl)	Ultrafiltration	Cheong et al. (2009)
PEG precipitation (10 % PEG8000+ 0.3 M NaCl)	–	Sánchez et al. (2012)
PEG precipitation (10 % PEG8000+ 0.3 M NaCl)	Chloroform-butanol	Summa et al. (2012)
PEG precipitation (10 % PEG6000+ 0.3 M NaCl)	Chloroform: butanol	ISO/TS 15216-1 (2013), ISO/TS 15216-2 (2013)
Ultracentrifugation at 240,000 × g/1 h	–	Kurdziel et al. (2001)
Ultracentrifugation at 200,000 × g/2 h.	–	Le Guyader et al. (2004b), Rutjes et al. (2006)
Ultracentrifugation at 235,000 × g/2 h	–	Rzeżutka et al. (2005), Rzeżutka et al. (2006)
Ultracentrifugation using centrifugal filter (100 K NMWL)	–	Butot et al. (2007)
Ultracentrifugation at 50,000 × g/3 h 20 min	–	Summa et al. (2012)
Ultrafiltration	–	Cliver et al. (1983)

(continued)

Table 9.5 (continued)

Concentration method	*Secondary concentration step*	*Reference*
Ultrafiltration using Amicon filters	–	Le Guyader et al. (2004b), Rutjes et al. (2006)
Ultrafiltration using Vivaspin concentrator 50,000 MWCO (Sartorius, Goettingen, Germany)	–	Scherer et al. (2010)
Ultrafiltration using a micro-concentrator device	–	Summa et al. (2012)
Ultrafiltration device (Amicon Ultra-15)	–	Brassard et al. (2012)
Filter adsorption—elution using glass fibers, melanin—impregnated paper, epoxy pleated cartridge filter	Organic flocculation by 3 % beef extract	Ward et al. (1982)
Filter adsorption-elution on Zetaplus 60 S filter for virus adsorption	Ultrafiltration using an Amicon centrifugal filtration (5000 × g/10 min)	Brassard et al. (2011)
Filter adsorption-elution on 0.45 μm negatively charged membrane Stericup filter (250 ml)	Ultrafiltration step using a Centriprep Concentrator® 50 (Nihon, Millipore)	Corrêa and Miagostovich (2013)
Concentration by magnetic beads coated with virus-specific ligands (using HBGA-MB)	–	Morton et al. (2009)
Concentration by magnetic beads coated with virus-specific ligands	–	Summa et al. (2012)
Concentration by magnetic beads coated with virus-specific ligands (using PGM-MB)	–	Pan et al. (2012)
Anion exchange on amorphous calcium phosphate(ACP) particles	–	Shinohara et al. (2013)

PEG polyethylene glycol, *HBGA-MB* histo-blood group antigen-conjugated beads-conjugated magnetic beads, *PGM-MB* porcine stomach mucin-conjugated magnetic beads

let containing the virus is eluted in a small volume of a suitable alkaline buffer. The alkalinity of the elution buffer is important to detach the virus from the flocculate by changing the net charge on its surface to negative instead of positive. Beef extract (3 % solution at pH 3.5) was used as an organic flocculent to concentrate feline calicivirus (FCV) eluted from lettuce and strawberry samples followed by elution of the virus from the flocculate by using PBS buffer at pH 9.0 (Gulati et al. 2001). Recently NoV and MNV eluted from strawberry samples were concentrated by this technique using skim milk as an organic flocculent followed by re-suspension of the pellet in PBS pH 7.5 (Melgaço et al. 2016).

Polyethylene Glycol (PEG) Precipitation Polyethylene glycol precipitation is the most commonly used method for virus concentration. It is based on using PEG as an aqueous precipitant for precipitating the eluted viruses; PEG is known to reduce the solubility of macromolecules (Lewis and Metcalf 1988). After separation of the precipitated virus by centrifugation, the virus pellet is re-suspended in a suitable buffer. This method allows the precipitation of viruses at neutral pH and at high ionic concentrations without precipitation of other organic material (Stals et al. 2012). It is important, therefore, to adjust the pH of the virus eluate to 7.0 ± 0.2 before adding PEG. Different molecular weights of PEG are available but the most commonly used are 6000 and 8000; there was no significant difference in recovery of NoV from strawberries or raspberries when PEG molecules from 6000–20,000 Da were used (Kim et al. 2008). The PEG is usually added in final concentrations of 6–30 % w/v of the virus eluate. Sodium chloride (0.3 M) is added to provide a high ionic strength needed for PEG precipitation (Baert et al. 2008; Love et al. 2008; Cheong et al. 2009; Sánchez et al. 2012; Summa et al. 2012). In some studies, higher concentrations of NaCl have been used e.g., 1.2 M (Le Guyader et al. 2004a) and 1.5 M (Dubois et al. 2002; Dubois et al. 2007). The advantages of PEG-based concentration methods are that they are rapid, inexpensive, and nondestructive of virus (Lewis and Metcalf 1988). The only disadvantage is its need to neutralize the pH of the virus eluate before adding PEG (Stals et al. 2012).

Ultracentrifugation In this procedure, very high centrifugal speed is used to sediment the viral particles from clarified virus eluates without the need for filtration or flocculation (Croci et al. 2008). After sedimentation of virus particles, the supernatant is decanted and the pelleted virus is re-suspended in a small volume of suitable buffer (Kurdziel et al. 2001). Several different ultracentrifugation speeds and times have been used. As shown in Table 9.5, the centrifugal speeds have ranged from $50{,}000 \times g$ to $240{,}000 \times g$ and time of ultracentrifugation has ranged from 1 h to 3 h 20 min (Kurdziel et al. 2001; Le Guyader et al. 2004b; Rutjes et al. 2006; Rzeżutka et al. 2005; Rzeżutka et al. 2006; Summa et al. 2012). Additional purification of virus eluates is required if subjected to ultracentrifugation protocols because debris and other components originating from the food samples can sediment simultaneously with

virus particles and interfere with their detection/isolation. The purification can be performed by either high speed conventional centrifugation or 0.22/0.45 μm filtration. Compared to the other concentration techniques, the main advantage of this technique is that it is very consistent (Stals et al. 2012). On the other hand, it has several disadvantages such as: (1) the need for expensive equipment, (2) the need for elimination of (hard) fruit/vegetable matter from the final samples, and (3) the resultant voluminous pellets that can occasionally be difficult to dissolve (Croci et al. 2008; Stals et al. 2012).

Ultrafiltration Unlike filter-adsorption elution methods, ultrafiltration concentrates viral particles by entrapping them from the eluate on the basis of molecular weight rather than by particle charge (Croci et al. 2008; Stals et al. 2012). The filter membranes have pore sizes of 50–100 kDa allowing only the passage of liquids and particles with less than 50–100 kDa molecular mass. Viruses have molecular mass over this limit so they are captured by the filters (Le Guyader et al. 2004b). When ultrafiltration is used for virus concentration, the eluate needs to undergo an efficient clarification and purification process to avoid clogging of the filter. The reported advantage of ultrafiltration is that PCR inhibitory components are completely eliminated (Rutjes et al. 2005). In the past, expensive filter holders with magnetic stirrers and high pressure were needed for ultrafiltration. Currently, ultrafiltration procedures use spin columns or microconcentrators to concentrate 1–80 mL of eluate to final volumes of 25–200 μl (Croci et al. 2008). In some studies, PEG precipitation provided better virus recovery than ultrafiltration. For example, recovery of NoV from lettuce, raspberries, and ham with ultrafiltration was 9, 3 and 7 %, while virus concentration by PEG resulted in 23, 7 and 24 virus recovery, respectively (Scherer et al. 2010). An ultrafiltration-based technique was used to concentrate virus eluted from fresh strawberries, frozen raspberries, frozen blueberries, and fresh raspberries, resulting in recoveries from 1.7 to 19.6 % (Butot et al. 2007). It has been reported that virus recovery by ultrafiltration can be somewhat increased by treating the filters with bovine serum albumin (BSA) or by sonication of the purified virus eluate (Jones et al. 2009).

Filter Absorption-Elution In this technique negatively charged filters are used to capture the positively charged viral particles from the eluate after adjusting the pH to about 3.5. The acidic pH is necessary for maintaining the viral particles positively charged. After capturing the virus on the filter, a small volume of an alkaline elution buffer is used for eluting the virus by changing its charge. Various filters have been used such as glass fiber, melanin-impregnated paper, epoxy pleated cartridge filters (Ward et al. 1982), Zeta plus 60 S filters (Brassard et al. 2011), and 0.45 μm negatively charged membrane (Stericup) filters (Corrêa and Miagostovich 2013). This technique requires that an efficient clarification process for the eluate be used prior to filtration to avoid clogging of the filter. Sometimes an acid rinse of the filter (with 5 mM H_2SO_4, pH 3.0) is needed to remove cations from the filter (Corrêa and Miagostovich 2013).

Concentration by Amorphous Calcium Phosphate (ACP) Amorphous calcium phosphate (ACP) particles are composed of hydroxyapatite precursors, spherically shaped with multiple pores. The phosphate ions, calcium ions, and hydroxide ions in ACP particles are more uniformly distributed than those in hydroxyapatite. The ACP particles are thought to interact with electrically-charged substances due to the effects of ion exchange or electrostatic attraction. Unlike adsorption on filters, in this method the alkaline virus eluate does not need to be neutralized or adjusted to an acidic pH since the negatively charged virus particles in the alkaline eluate are replacing the negatively charged ions covering the ACP particles, according to the principles of the process of anion exchange. Practically, ACP particles are added to the eluate and stirred for 1 h at room temperature and then collected by centrifugation. The pellet of ACP-virus particles is suspended in a small volume of a suitable buffer. This technique was used successfully for recovery of FCV and NoV from cabbage, lettuce and ham and resulted in 32–55 % recovery of FCV and 12–57 % recovery of NoV (Shinohara et al. 2013).

Concentration by Using Magnetic Beads Coated with Virus-Specific Ligands This is a very specific method for concentration of certain viruses from the eluate. For concentration of NoV, magnetic beads coated with either histo-blood group antigen (HBGA) types A, B, H(2) and H(3) or type III porcine gastric mucin are used so as to bind NoV particles after elution from food samples. Subsequently, NoV particles are eluted from the magnetic beads using an appropriate buffer (Morton et al. 2009; Pan et al. 2012; Summa et al. 2012). Another type of magnetic bead covered with monoclonal (K3-2F2) antibodies against HAV has been used for HAV concentration (Bidawid et al. 2000). The concentration methods using magnetic beads coated with virus-specific ligands were used successfully for concentrating NoV and HAV from lettuce, green onions, strawberries, and ham (Bidawid et al. 2000; Morton et al. 2009; Pan et al. 2012; Summa et al. 2012). GI and GII NoV genogroups were recovered from fruit and vegetable salad, blueberries, and tomatoes with virus recoveries of 6–30 % (Pan et al. 2012). Specific virus extraction from various food types and efficient removal of PCR inhibitors are the main advantages of this technique. However, questions could be raised about the long-term use of monoclonal antibodies due to the immunogenetic drift of these food-borne viruses (Stals et al. 2012).

3.1.4 Secondary Concentration Step

A secondary concentration and/or purification step can be performed after concentration for: (1) removal of interfering substances previously co-purified with viral particles (Dubois et al. 2002; Dubois et al. 2007; Baert et al. 2008; Love et al. 2008; Scherer et al. 2010; Summa et al. 2012) and (2) for further reducing the final volumes of viral concentrates (Ward et al. 1982; Cliver et al. 1983; Schwab et al. 2000; Baert et al. 2008; Brassard et al. 2011; Corrêa

and Miagostovich 2013). In Table 9.5, the main concentration methods and the secondary concentration/purification steps are presented. For the first purpose, some of the methods of clarification previously discussed are used, such as using Chloroform-butanol and Freon or Vertrel, while organic flocculation, PEG precipitation and ultrafiltration were used as a secondary concentration procedure for the second purpose (Table 9.5).

3.2. The Approach of Direct Recovery of Viral RNA from Food

Direct recovery of viral RNA is a new approach for detection of viruses in foods. It involves treatment of foods with guanidinium isothiocyanate (GITC)/phenol based reagent, followed by purification of the extracted RNA. Direct RNA extraction was applied successfully on fat/protein-based foods. One to 10^2 NoV RT-PCR units could be recovered from 10 to 30 g of hamburger, turkey, roast beef, penne, tagliatelle, and deli ham (Schwab et al. 2000; Baert et al. 2008; Perrin et al. 2015). Detection of 10^6 NoV GI/GII genomic copies was consistently possible for a variety of ready-to-eat foods, while 10^4 NoV GI/ GII genomic copies could occasionally be detected (Stals et al. 2011).

4. QUALITY ASSURANCE OF VIRUS RECOVERY METHODS FROM FOOD

The complexity of food matrices and the low number of viruses often present in foods make virus recovery a complex and difficult process. Furthermore, the recovered virus concentrates may contain inhibitors of nucleic acid amplification by PCR. Incorrect performance of any step during the virus recovery procedure or the molecular assay may lead to false negative results. Therefore, it is necessary to follow a quality assurance procedure to facilitate the determination of the method's efficiency of detection and to evaluate its performance or identify where the method has failed to perform correctly in order to discriminate between false-negative and true-negative results. Quality assurance, which is critical when recovering and detecting viruses from food samples, means the use and evaluation of adequate controls throughout the different procedural steps (Diez-Valcarce et al. 2011; Stals et al. 2012). Diez-Valcarce et al. (2011) recommended the use of two controls e.g., a sample process control (SPC) and an internal amplification control (IAC) to distinguish between failed recovery and failed detection of viruses in foods. The IACs have been widely used in the majority of current investigations (Hoorfar et al. 2004; Diez-Valcarce et al. 2011; Martínez-Martínez et al. 2011; Rodríguez-Lázaro et al. 2004, 2006), but only a few investigations have described the use of SPCs to show the effect of the food matrix on the efficiency of a virus recovery process (Croci et al. 2008). The use of IACs is discussed in more details in Chap. 11. The SPC consists of adding a control virus to the sample in

one of two ways. (1) adding it to the same sample as internal process control (IPC) or (2) adding it to a sample analyzed in parallel to the sample of interest, as an external process control (EPC) (Croci et al. 2008).

The essential characteristics of the SPC viruses are: (1) they should be structurally and genetically similar to the tested virus, (2) have a similar route of infection, (3) should be easy to cultivate, and (4) should not be a natural contaminant for the tested food sample. The viruses that have been most frequently been used as SPCs are MNV-1, FCV, a genetically modified mengovirus (vMC0), and the MS2 bacteriophage. The canine calicivirus (CaCV) and poliovirus (PV) are less commonly used. The MNV-1 (genogroup V NoV) was proposed as a SPC when detecting human NoV because of the genetic similarities of the two viruses (Wobus et al. 2006; Cannon et al. 2006; Diez-Valcarce et al. 2011). The FCV was used as an SPC when detecting NoV in shellfish, bottled water and fresh produce (Kingsley 2007; Mattison et al. 2009; Schultz et al. 2010; Uhrbrand et al. 2010). A genetically modified strain of mengovirus, which is not pathogenic to humans and can be cultivated in HeLa-cells, was used as an SPC control for the detection of NoV and HAV in shellfish and bottled water (Hahn and Palmenberg 1995; Costafreda et al. 2006; da Silva et al. 2007; Comelli et al. 2008; Uhrbrand et al. 2010).

5. CONCLUSIONS

The development of efficient virus detection methods from food is necessary for epidemiological investigations to study the fate of viral pathogens in foods, to investigate possible strategies for prevention of foodborne viral contamination, and to routinely test foods for food safety assurance. Unfortunately, so far there is no standard method that can be generally applied for all types of foods because of the complexity and diverse characteristics of foods implicated in foodborne viral outbreaks. In this chapter, we have summarized the different approaches and protocols used for virus recovery from foods.

Generally, for detection of viruses in food a three-step strategy is used consisting of: (1) virus recovery, (2) extraction of viral nucleic acids, and (3) molecular detection of the viral RNA. Two main approaches are used to recover viruses from foods: (1) virus particles can be recovered intact, or without disrupting the viral capsid, or (2) viral RNA can be directly recovered. Most of the virus particle recovery approaches follow a three-step protocol (Table 9.2, Fig. 9.1) consisting of: (1) elution of virus particles from the food matrix, (2) clarification of the virus-containing eluate, and (3) concentration of the recovered virus particles in a small final sample volume. Different elution buffers can be used, but the most frequently used is an alkaline buffer with a pH ranging from 8 to 10.5 to elute the viral particles from the surface of fruits and vegetables. The alkaline pH allows the viruses to desorb from food surfaces. In a majority of protocols, the elution buffers are used in combination with spe-

cific supplements that are added to enhance the recovery of viral particles from foods. In addition, beef extract and glycine are often used to reduce non-specific virus adsorption to the food matrix during the elution step. In eluting virus from foods, a mechanical force often needs to be applied to help wash the virus off the food sample into the elution buffer.

After obtaining the virus eluate, a clarification step is used to remove PCR inhibitors and other impurities that may hinder the next concentration step by clogging the filter membranes. In general, the clarification step must be done under alkaline conditions (high pH) to prevent the re-adsorption of viruses to the fruit or vegetable matter. The most commonly used method for clarification of viral eluate is the use of one-step low speed centrifugation. Cat-Floc™ and bentonite are flocculents that are added to the eluate for enhancing the separation of the remaining food matter. Freon and its substitute "Vertrel" or a mixed organic solvent (chloroform-butanol; 1:1 v/v) are added to the virus eluate before centrifugation as an organic solvent to remove PCR inhibitory substances.

After clarification of the virus eluate, the virus must be concentrated to make it detectable by the molecular methods. Several techniques can be employed such as organic flocculation, PEG precipitation, ultrafiltration, ultracentrifugation, filter adsorption-elution, adsorption-elution on amorphous calcium phosphate (ACP) particles and separation using magnetic beads coated with virus-specific ligands. A secondary concentration and/or purification step can be performed after concentration for the removal of interfering substances previously co-purified with viral particles and for further reducing the final volumes of viral concentrates. Recently, the use of a sample process control has been recommended to determine the method's efficiency of detection and to evaluate the performance of the recovery procedure and help to discriminate between the false and true negative results.

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CHAPTER 10

Methods for Virus Recovery in Water

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1. INTRODUCTION

Research involving the recovery of viruses from various water sources can be traced back to the 1950s when scientists were eager to better understand the occurrence and transmission of disease-causing viruses in water; most significant viruses at the time were poliovirus and hepatitis virus. LoGrippo and Berger (1952) utilized ion-exchange resins to concentrate poliovirus, and shortly thereafter Kelly (1953) demonstrated the use of these same resins for the concentration of coxsackie viruses from sewage samples. This seminal research led to development of the first methods that relied on manipulation of virus surface charge for recovering viruses from large volumes of water. Virus adsorption to filter media followed by elution by a pH-adjusted solution (Wallis and Melnick 1967) became collectively known as VIRADEL (virus adsorption elution) method. Shortly after VIRADEL methods were introduced, alternative methods to concentrate viruses based on size exclusion (i.e. ultrafiltration based on nominal molecular weight cut-off) were investigated. Belfort et al. (1975) concentrated viruses from 5 L of water using hollow fiber membranes and from this initial report through the present day, numerous studies have shown hollow-fiber ultrafiltration is effective for the recovery of viruses from water (Fong and Lipp 2005; Gensberger and Kostić 2013; Hill et al. 2007).

At this point, two questions arise: (1) How are viruses in water related to viruses in food and (2) Why is it important? Simply stated, there is an intimate connection between food safety and water quality as water is used at almost every node in the food production process. Moreover, viruses are the primary cause of foodborne disease outbreaks (FBDO) in the United States and in most other high-income countries (EFSA BIOHAZ Panel 2011; Lopman et al. 2003; Scallan et al. 2011). From an epidemiological perspective, human noroviruses (HuNoVs) are the most significant in foodborne trans-

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mission though other viruses of concern include hepatitis A virus (HAV), enteroviruses, and hepatitis E virus (HEV; Le Guyadar et al. 2008; Yugo and Meng 2013).

The most commonly reported connections between water quality and food safety are viruses in fresh produce and shellfish (i.e. bivalve mollusks such as oysters) (see Chaps. 6 and 7 in this book). For these foods, large volumes of water must be used during pre- and post-harvest processing. The ongoing conversation in shellfish production revolves around the relevance of using fecal indicator bacteria (*Escherichia coli*, *Enterococcus* spp.) as predictors of viral pathogen contamination when monitoring the quality of harvesting waters or shellfish meats (Richards et al. 2010). The issue arises because an indicator should correlate with the presence of pathogens; however, bacterial indicators have been repeatedly shown to correlate poorly with viruses (Field and Samadpour 2007). Fresh produce (i.e. fruits and vegetables) can become contaminated with waterborne bacteria and viruses during production via irrigation water, preparing and spraying pesticides, preventing dehydration, or during produce washing or cooling. The proposed agricultural water standards in the US FDA's proposed rule for produce safety rely on *E. coli* to indicate sanitary quality of water (USFDA 2014). Thus, the same limitations would apply regarding the often observed absence of a relationship between bacterial indicators and human pathogenic viruses.

Human enteric viruses may be introduced into the water environment through various routes including discharge of sewage-contaminated water into food production settings (i.e. oyster harvesting waters and surface water used for irrigation), land application of municipal biosolids, wastewater, or septage with subsequent runoff, and groundwater contaminated by infiltrating surface water, faulty septic systems or leaking sanitary sewers (Gibson 2014). Overall, data are limited on virus occurrence in water used for food production even though the industry's heavy reliance on water suggest it is a vehicle for dissemination of viruses in the food supply (Song et al. 2006; Stine et al. 2005).

There are several options for recovering viruses from water, although each method brings along its own set of challenges, primarily stemming from the low but significant levels of human enteric viruses that may be present in environmental water samples at sites located around the world (Dong et al. 2010; Gibson et al. 2012; Sinclair et al. 2009; World Health Organization 2009; Ye et al. 2012).

2. VIRUS RECOVERY METHODS

Wallis et al. (1979) originally prescribed the ideal method for virus recovery as being able to: (1) process large volumes (>100 L) of various types of water in the least amount of time; (2) consistently concentrate most types of viruses

present in water and wastewater, (3) be used easily and economically, and (4) recover viruses that are particle-associated or present in viral aggregates. In the subsections that follow, various methods are described for recovering and concentrating viruses from water.

2.1. VIRADEL

The first generation of VIRADEL methods was based on adsorption of viruses to electronegative (EN) membrane filters (Sobsey et al. 1973). However, because viruses have a net negative charge on their capsid surface at neutral pH, to facilitate virus adsorption to the negatively-charged filters, EN-based methods require cumbersome chemical modification of the water sample including acidification or addition of multivalent cations (Sobsey and Jones 1979). Shortly thereafter, Sobsey and Jones (1979) suggested that membrane filters with a more positive charge at the pH of natural waters and tap water (pH 5–9) would be more advantageous for virus recovery compared to EN-based methods. This initial investigation of electropositive (EP)-based membrane filter methods by Sobsey and Jones (1979) demonstrated a marked increase in virus recovery from tap water (e.g., <5 % recovery with EN filter vs. 64 % recovery with EP filter) and served as the basis for developing the standard method for virus recovery and concentration from large volumes of water (USEPA 2001).

Wallis et al. (1979) identified several factors influencing the adsorption of viruses to filter surfaces: filter surface charge and surface area resulting from the composition and design of the filter membrane; flow rate during sampling (i.e. high flow rate equates to less viruses adsorbed); ratio of pore diameter to virus diameter; pH; multivalent cations; and the presence of proteinaceous substances competing with viruses for adsorption. Filter surface charge plays a significant role in virus adsorption as demonstrated by the difference in viral recovery between EN- and EP-based methods. In addition, virus adsorption capacity decreases with decreasing filter surface area because there are a finite number of adsorption sites per unit area (Wallis et al. 1979). However, additions of acids (for pH manipulation) and salts (multivalent cations) can greatly increase filter adsorption capacity regardless of filter surface area; therefore, the virus adsorption capacity of filters is really never exceeded (Wallis et al. 1979). The impact of multivalent cations on filter charge is one of the primary reasons why EN-based methods outperform EP-based methods for the recovery of viruses from marine waters (Katayama et al. 2002; Lukasik et al. 2000). Overall, the net charge of the filter is one of the primary determining factors in virus adsorption.

Present day, researchers still use both EN- and EP-based methods for recovery of viruses from water though far more emphasis has been placed on EP-based methods. Table 10.1 summarizes the VIRADEL methods that are most commonly utilized for recovery of viruses, and Table 10.2 provides representative recovery efficiencies for each VIRADEL method by water type. For the EP-based methods, beef extract—glycine solutions at an alkaline pH is

most often used for virus elution from the filter membrane (Table 10.1). However, other eluents have previously been used including 5× nutrient broth (Hill et al. 1974), tryptose phosphate broth (Farrah et al. 1976), and amino acids—arginine and lysine (Farrah and Bitton 1978). The type of eluent is important to note as organic and inorganic compounds in beef extract solutions are known to inhibit reverse transcription (RT) for cDNA synthesis from viral RNA as well as PCR amplification (Abbaszadegan et al. 1993). This is important because many waterborne viruses are not readily culturable requiring downstream detection methods that rely on RT and PCR technologies.

After filtration with EN-based methods the filter is rinsed with a dilute acid followed by elution with alkaline buffer (pH > 9.0) containing either high salt concentration and surfactant (Hamza et al. 2009) or proteinaceous substances such as skimmed milk (Wyn-Jones et al. 2011). De Keuckelaere et al. (2013) compared four methods, two EN-based, one EP-based, and one using tangential flow filtration for recovery of five types of human enteric viruses from four irrigation water sources and processing water from a fresh cut lettuce processing plant. The investigators concluded the EN-based method including virus elution with high salt concentration buffer provided the highest virus recoveries across the range of water types tested. However, De Keuckelaere et al. (2013) reported much lower recovery efficiency for three of the four methods evaluated as compared to previously published reports.

A novel filter-less method was reported by Calgua et al. (2008) where 5–10 L volumes of seawater or freshwater amended with artificial sea salts are flocculated with 1 % (w/v) skimmed milk solution at pH 3.5. Samples are stirred for 8 h, the floc is allowed to settle for another 8 h, and then centrifuged. Mean virus recoveries from freshwater were in the range of 40–50 % (Calgua et al. 2013). The EN-based methods and skimmed milk method may be considered advantageous when compared to EP-based methods and ultrafiltration based on the volume of water needed. For instance, only 2–10 L (Fong et al. 2005; Lee and Kim 2008; Hamza et al. 2011) of a given water sample can be concentrated to detect viruses in the former methods; however, given the same water sample, hundreds of liters may need to be concentrated by EP-based and ultrafiltration to detect the viruses present. Simultaneous concentration of PCR inhibitors and particulates during large volume filtration is speculated to offset the benefits of concentrating more viruses (Albinana-Gimenez et al. 2009; De Keuckelaere et al. 2013), but why small sample volumes appear to provide the same limits of virus detection has not been systematically investigated. Confounder effects, such as correlation between the method selected for sampling and virus concentrations in the waters being sampled, or between sampling method and the use of nested PCR for virus detection, are possible considerations.

The EP-based filtration method described by Sobsey and Jones (1979) served as the basis for USEPA to prescribe using the positively-charged 1MDS filter for virus concentration from water for the Information Collection Rule (USEPA 2001). Although effective (Table 10.2), the 1MDS method has

Table 10.1 VIRADEL (Virus Adsorption—Elution) methods for recovery of viruses from water samples

	VIRADEL filtration methods				
	1MDS[a]	NanoCeram®[b]	N66 Posidyne	Glass wool	HA—membrane
Principle	EP	EP	EP	EP	EN
Water type[c]	S, G, D, E, W	S, G, D, E	S, D	S, G, D, E, Ag, W	S, G, D, E
Volume (L)[d]	1–>1000	10–>1900	20–>200	2–>100,000	0.2–20
Sample/filter amendments	Adjust sample pH if >8.0	None	None	Adjust sample pH if >7.5	Sample pH 3.5–4.0 Or Precondition filter
Pre-filter	Optional	Optional	Optional	Optional	Optional
Elution	Beef extract—glycine (pH 9.5)	Beef extract—glycine (pH 9.0–9.5); sodium polyphosphate—glycine (pH 9.3)	Beef extract (pH 9.5)	Beef extract—glycine (pH 9.5)	Tr alk (pH 9.2); skimmed milk glycine solution (pH 9.5); TGBE (pH 9.5)
References	Cashdollar and Dahling (2006), Gilgen et al. (1997), Lee et al. (2011), Polaczyk et al. (2008), Rose et al. (1984), and Sobsey and Glass (1980)	Cashdollar and Wymer (2013), Gibbons et al. (2010), Ikner et al. (2011), Karim et al. (2009), and Lee et al. (2011)	Cashdollar and Dahling (2006), Shields et al. (1986)	Environment Agency, (2000), Francy et al. (2013), Gibson et al. (2012), Grabow and Taylor (1993), Lambertini et al., (2008, 2011), Millen et al., (2012), and Vilagines et al. (1997)	De Keuckelaere et al. (2013), Farrah et al. (1976), Haramoto et al. (2009, 2012); and Victoria et al. (2009)

EP electropositive, *EN* electronegative, *HA* mixed cellulose esters, *ND* not done; *Tr alk* 0.05 M KH_2PO_4, 1.0 M NaCl, 0.1 % (v/v) Triton X-100, *TGBE* 100 mM Tris, 50 mM glycine, beef extract 1 % (w/v); *S* surface water, *G* groundwater, *D* drinking water, *E* salt water, *Ag* agriculture runoff, *W* wastewater

[a]Zetapor® or Virosorb®

[b]Both cartridge and disc filter options are available, but only NanoCeram® Virus Sampler cartridge filters are referred to here. In addition, ViroCap is the name of the self-contained cartridge format of NanoCeram®

[c]As reported in published literature

[d]Range of water volumes for all water types; values based on published research

Table 10.2 Virus recovery efficiencies for VIRADEL filtration methods by water type[a,b]

	% Recovery[c]				
	Surface water	*Groundwater*	*Tap water*	*Seawater*	*Wastewater*
1MDS	36–100	62–72	33–67	40–53	14–61
NanoCeram®	38–65	86	14–84	<3–>96	ND
Glass wool	5–72	8–56	28–98	15–99	52–62
HA-membrane	15–67	ND	3–80	3–53	ND

ND not done

[a]References listed in Table 9.1 apply here as well

[b]Viruses tested include; adenoviruses (type 2, 40,41), human astroviruse GI, coxsackie virus B5, echoviruses (type 1, 7, 18), Hepatitis A virus, MS2 bacteriphage, murine norovirus, human norovirus (GI and GII), phi X174 bacteriophage, polioviruses (LSc, Mahoney, Sabin strains), human rotavirus

[c]Recovery percentages listed are based on primary concentration followed by detection of viruses by either culture-based or molecular-based methods

been criticized for being cost prohibitive for routine monitoring purposes—each filter cartridge costs approximately $200–300 (Cashdollar and Wymer 2013). In 2010, USEPA published Method 1615, a revised method for detecting enteroviruses and noroviruses in water that permits virus concentration by the positively charged NanoCeram® filter, which cost roughly 20 % ($40–60) of the cost of the 1MDS filter and is equally as effective for these two viruses (Fout et al. 2010). The Nanoceram filter does not appear to be as effective for concentrating adenoviruses (Gibbons et al. 2010; Ikner et al. 2011; Pang et al. 2012; McMinn 2013).

Glass wool filtration is another effective EP-based method (Table 10.1). Oiled sodocalcic glass wool is washed in series with 1 N HCl, distilled water, 1 N NaOH, and a final wash with distilled water until a neutral pH is achieved. The washed glass wool can be stored in sterile phosphate buffered saline for up to 1 week at 4 °C. Filters are made by packing washed glass wool tightly into column housings with size and fittings appropriate for the virus sampling plan at hand (Vilagines et al. 1993; Millen et al. 2012). Filter construction configurations are highly flexible for specific projects and can be used to filter large volumes of water (Gibson et al. 2012; Lambertini et al. 2008; Vilagines et al. 1997). Similar to other EP-based filter cartridges, glass wool requires the use of an alkaline eluent such as a beef extract—glycine solution. Glass wool filters are a fraction of the cost—$4.40 per filter (not including labor)—of NanoCeram® and 1MDS filters (Lambertini et al. 2008) and are demonstrated to achieve similar virus recovery efficiencies (Table 10.2). The primary disadvantage of glass wool filters is that they are not commercially available, lending to the perception that hand-packing in the laboratory may lead to filter-to-filter variability in virus recovery efficiency (Cashdollar and Wymer 2013). Even then, the United Kingdom Environment Agency utilizes the glass wool filtration technique in standard methods for the recovery and concentration of viruses in groundwater (Environment Agency 2000).

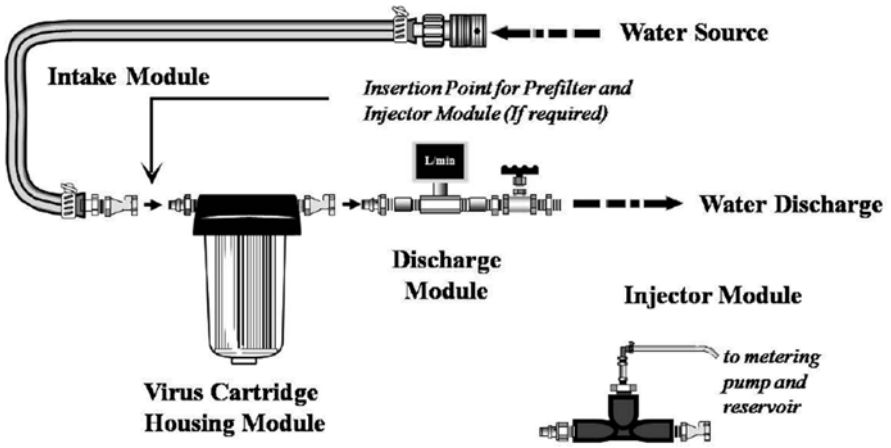
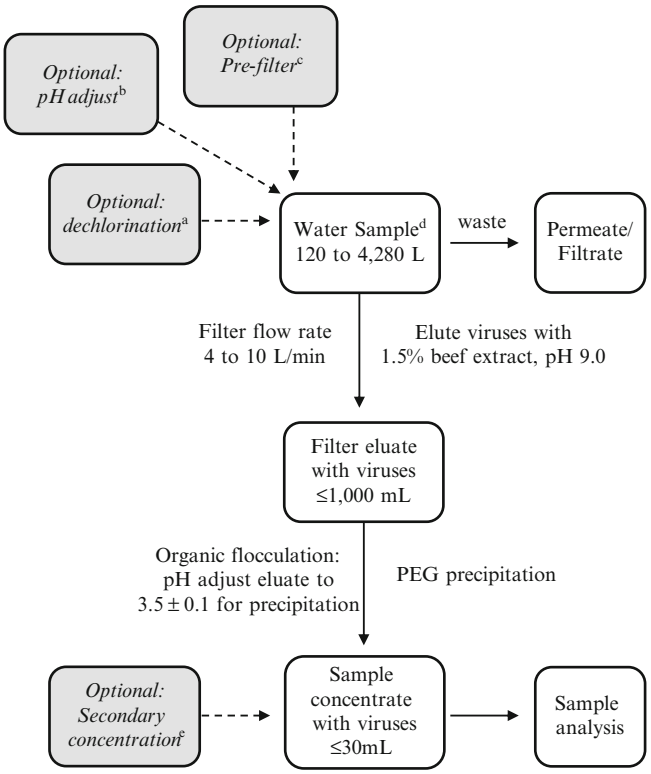


Fig. 10.1 Basic setup for recovery of viruses from water using electropositive (EP)-based methods. An injector module is used for dechlorination if disinfectant present in the water source and/or for pH adjustment. A 10-μm polypropylene prefilter may be required if the water sample turbidity is elevated.

As described in USEPA Method 1615, the basic setup and steps for recovering viruses from tap water using an EP-based cartridge filtration method are shown in Figs. 10.1 and 10.2, respectively. Overall, when using EP filters such as NanoCeram®, 1MDS, or glass wool, the steps taken to recover viruses from a given water sample will depend on the need to neutralize chlorine residual disinfectant, adjust pH to the optimal level for virus adsorption, and add a pre-filter for highly turbid samples.

2.2. Hollow Fiber Ultrafiltration

Hollow fiber ultrafiltration (HFUF) for virus concentration and recovery from water was initially investigated by Belfort et al. (1975). There was a renewed interest in this method a decade ago for its potential to concentrate not only viruses but also other classes of microorganisms. Ultrafiltration methods rely on size exclusion for virus concentration rather than adsorption and hence difficulties associated with adsorbing viruses to various filter media are avoided. Ultrafilters are rated by molecular weight cut-off (MWCO) ranging from 1 to 300 kilodaltons (kDa), corresponding to a nominal pore size between 5 and 35 nm (Pall Corporation 2015). There are many different shapes of ultrafilter membranes available including spiral-wound, flat sheets, flat discs, and hollow fibers; the latter is the most commonly used shape for waterborne virus concentration. The shape of the filter impacts the membrane surface area available for filtration. Most hollow fiber membrane filters used for recovery of viruses range from 1.3 to 2.5 m^2 (Table 10.2). Ultrafiltration membranes are constructed from a variety of polymers such as polysulfone, polyacrylonitrile, and cellulose triacetate and can be used in two different modes; tangential (cross) flow (TF) and direct (dead-end) flow (DE).



[Adapted from Fout *et al.* (2010) and Francy *et al.* (2013)]

Figure 10.2 Virus Adsorption—Elution (VIRADEL) filtration method procedure. [a]If the water sample has a disinfectant present based on measured chlorine residual, then it must be dechlorinated with 2 % sodium thiosulfate using the optional injector module (Fig. 10.1) prior to filtration. [b]If the water sample has a pH >9.0 or >8.0 for NanoCeram® and 1MDS Zetapor® filter cartridges, respectively, then the pH must be adjusted with 0.12 M HCl until it measures 6.5–7.5. [c] If the water sample turbidity is >20 NTU or >50 NTU for NanoCeram® and 1MDS Zetapor® filter cartridges, respectively, then a 10-µm polypropylene prefilter cartridge should be added prior to filtration but after dechlorination and pH adjustment. [d]The volume of water filtered depends on the water source and flow rate (Fout et al. 2010) [e]Various secondary concentration steps may be applied as described in Sect. 2.3 of this Chapter.

In TF filtration, a portion of the water (i.e. "feed" water) recirculates back to a central reservoir while the remaining portion passes through the membrane. The feed water is continuously recirculated until only a concentrate remains. Conversely, direct flow filtration pushes water through the membrane without recirculation, thus increasing the likelihood of membrane foul-

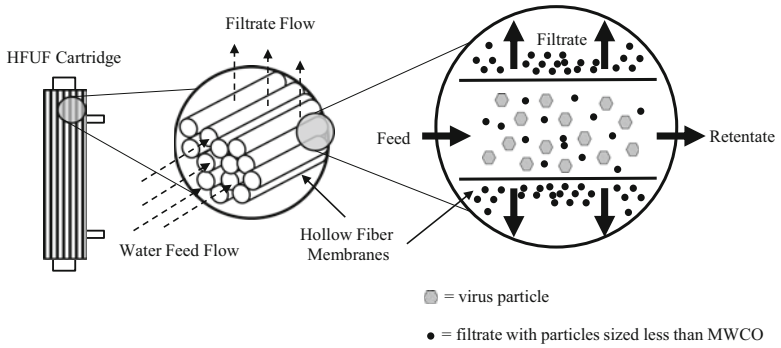


Figure 10.3 Diagram of tangential flow ultrafiltration using a hollow fiber membrane cartridge filter. *HFUF* hollow fiber ultrafiltration.

ing, or the loss of membrane permeability that occurs due to accumulation of aquatic substances on or inside the membrane (Huang et al. 2008). During TF-HFUF, an applied pressure serves to force a portion of the feed water through the membrane to the filtrate side while viruses are concentrated in the retentate as opposed to being filtered out (Fig. 10.3). In TF, the retained components do not build up at the surface of the membrane but instead are swept along by the cross directional flow, dramatically reducing fouling and decreasing the tendency for microbes to adhere to the filter surfaces (Huang et al. 2008; Morales-Morales et al. 2003).

In DE filtration, water is not recirculated but flows directly through the filter, retaining viruses and other particulates greater in size than the MWCO. Ultrafilters are attached directly to a pressurized water source (e.g. drinking water distribution system), or in unpressurized systems (e.g. irrigation canal), a peristaltic pump is used. Input pressure less than 20 lb/in^2 is recommended (Mull and Hill 2012; Smith and Hill 2009). Dead-end HFUF may be advantageous in field settings where set-up of TF-HFUF equipment can be cumbersome and filter clogging is not problematic as in low turbidity water.

Belfort et al. (1975, 1983, 1985) initially investigated ultrafiltration for recovery of viruses in water in the mid-1970s to 1980s and interest was renewed in the 1990s with the potential ultrafiltration afforded for recovering multiple classes of microorganisms (pathogenic viruses, bacteria, and protozoa) with a single sampling method. Kfir et al. (1995) first demonstrated the simultaneous recovery of viruses and protozoa from 10 L water samples using 50 kDa MWCO ultrafiltration disc membranes. Moreover, the study showed that UF provided higher, more consistent recovery of enteric viruses (e.g., 63–100 % with an average of 82 %) when compared to traditional VIRADEL methods.

Additional studies by Morales-Morales et al. (2003) and Hill et al. (2005) switched the focus to hollow fiber ultrafilters operated in tangential flow for

the recovery and concentration of viruses in water. In Morales-Morales et al. (2003), two different hollow fiber ultrafilters with 50 kDa MWCOs were evaluated to recover microorganisms in 2 and 10 L water samples (tap water, groundwater, and surface water). Reported recoveries for PP7 and T1 bacteriophage were 31–74 % for 10 L surface water samples (Morales-Morales et al. 2003). Similarly, Hill et al. (2005) described a TF-HFUF method for the simultaneous recovery of microbes in tap water using commercially available dialysis filters with hollow fiber membranes (Table 10.3). The process was optimized through addition of (1) sample amendments such as sodium polyphosphate (NaPP); (2) an alternative membrane blocking procedure (e.g., NaPP solution as opposed to calf serum as described in Morales-Morales et al. (2003)); and (3) a backwashing step with a solution containing Tween 80 (Hill et al. 2005). Polyphosphates increase the negative surface charge of microbes suspended in water, creating surface charge repulsion. Tween 80 serves as a surfactant for reducing hydrophobic interactions between the filter surface and microbes, reducing adsorption of microbes to the hollow fiber membrane surface. Average recovery efficiencies for viruses using the optimized TF-HFUF method were 91 and 49 % for MS2 bacteriophage and echovirus 1, respectively (Hill et al. 2005).

Shortly thereafter, Hill et al. (2007) published another study on the concentration of viruses from 100 L samples of tap water. This study is the foundation for the development and optimization of additional TF-HFUF methods for concentration of viruses from 100 L water samples (Polaczyk et al. 2008; Hill et al. 2009; Francy et al. 2009; Gibson and Schwab 2011; Rhodes et al. 2011; Liu et al. 2012; Wu et al. 2013). Based on these studies, the basic steps for recovering viruses and other classes of microbes from water using HFUF are shown in Fig. 10.4, and the configuration for TF-HFUF is shown in Fig. 10.5a.

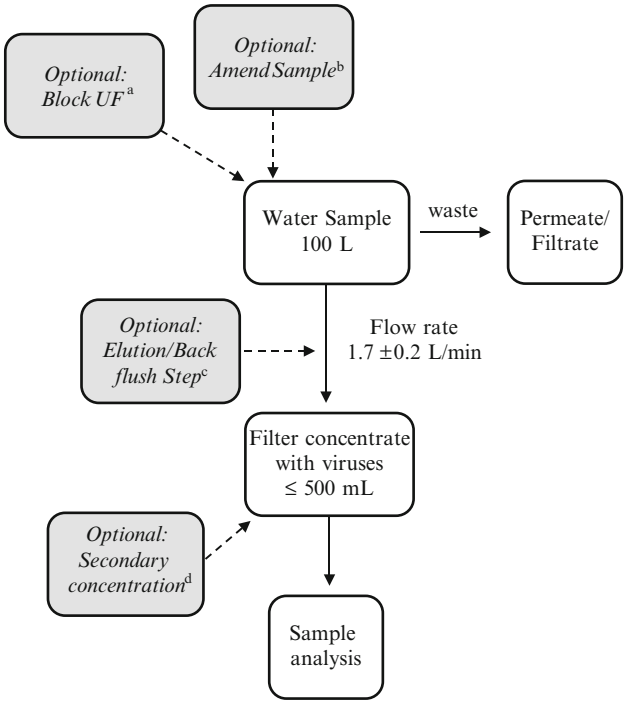
DE-HFUF methods gained attention due to the relative ease of field deployment compared to the TF-HFUF setup requirements. Olzewski et al. (2005) initially compared DE-HFUF to tangential flow ultrafilter cassettes (i.e. not hollow fiber dialyzers) and demonstrated similar recovery efficiencies (>65 %). However, high turbidity samples caused membrane fouling (or clogging) of the DE-HFUF (Olzewski et al. 2005). Smith and Hill (2009) demonstrated the recovery of diverse microbes including viruses from 100 L tap water and from mock surface water samples (i.e. tap water spiked with surface water to obtain a turbidity of 5 NTU) using DE-HFUF. The reported recovery efficiencies using DE-HFUF were similar to TF-HFUF (Table 10.4), making DE-HFUF an acceptable option for field-based sampling efforts. Additionally, Smith and Hill (2009) determined that Asahi Kasei REXEED 25S ultrafilters were better suited for use in DE-HFUF set-ups based on their hydraulic performance (i.e. ability to maintain an acceptable permeate flow rate at consistent, low system pressures) as compared to Exeltra Plus 210, F200NR, and REXEED 21S filters (Table 10.3).

Mull and Hill (2012) also investigated DE-HFUF for recovery of viruses from medium (approx. 50 NTU) to high (approx. 100 NTU) turbidity surface

Table 10.3 Hollow fiber ultrafiltration membranes used for virus recovery[a]

Vendor	*Model*	*MWCO (kDa)*	*Membrane composite*	*SA (m^2)*	*Fiber ID (μm)*	*References*
Asahi Kasei	Rexbrane REXEED™-25S	30	Polysulfone	2.5	185	Francy et al. (2009, 2013), Knappett et al. (2011), Mull and Hill (2012), Rhodes et al. (2011), Smith and Hill (2009), and USEPA and CDC (2011)
Baxter	Exeltra Plus 210	70	Cellulose triacetate	2.1	200	Gibson and Schwab (2011), Liu et al. (2012), and Smith and Hill (2009)
Fresenius Medical	F200NR	30	Polysulfone	2.0	200	Hill et al. (2007, 2009, 2010), Liu et al. (2012), Polaczyk et al. (2008), and Smith and Hill (2009)
	F80A	30	Polysulfone	1.8	200	Hill et al. (2005) Polaczyk et al. (2008), Wu et al. (2013)
Medivators Inc.	Hemocor HPH® 1400	65	Polysulfone	1.3	200	Francy et al. (2009), Liu et al. (2012)
Pall Corp.	Microza (AHP-0013)	50	Polyacrylonitrile	0.017	800	Morales-Morales et al. (2003), Rajal et al. (2007), and Winona et al. (2001)

MWCO molecular weight cut-off, *SA* surface area, *ID* inner diameter
[a]Table adapted from Holowecky et al. (2009)



[Adapted from Francy et al. (2013)]

Fig. 10.4 Hollow fiber ultrafiltration (HFUF) method steps. *UF* ultrafilter. [a]The ultrafilter may be blocked to reduce the potential for virus adsorption to the membranes; however, the need for blocking may depend on the brand of ultrafilter and water type (Liu et al. 2012). Blocking procedures may include (1) overnight incubation with 5 % calf serum (Hill et al. 2005); (2) recirculation of 500 ml 5 % calf serum for 5 min through the UF; (3) recirculation of 500 ml of blocking solution containing 0.055 % Tween 80, 0.001 % Antifoam A, and 0.1 % NaPP through the ultrafilter for 5 min (USEPA and CDC 2011); or (4) filtration of 1 L 0.1 % NaPP through the UF at 1.7 L/min with no back pressure (Hill et al. 2005). [b]Sample may be amended with sodium polyphosphate (NaPP) to a final concentration of 0.01 % prior to beginning filtration. [c]Following filtration, the ultrafilter can be eluted, or back flushed in the case of DE-HFUF, to capture any viruses that may be bound to the membrane. Elution buffers are recirculated within the TF-HFUF system and may include a solution containing 0.001 % Tween 80 or a solution containing 0.01 % Tween 80, 0.01 % NaPP, or 0.0001 % Y-30 antifoam emulsion. On the other hand, backflushing buffers are pumped through the permeate/filtrate port and may include solutions containing slightly higher concentrations of components found in the elution buffers used in TF-HFUF, such as 0.5 % Tween 80, 0.01 % NaPP, and 0.001 % Y-30 antifoam emulsion. [d]Various secondary concentration steps may be applied to the filter concentrate as described in Sect. 2.3 of this Chapter.

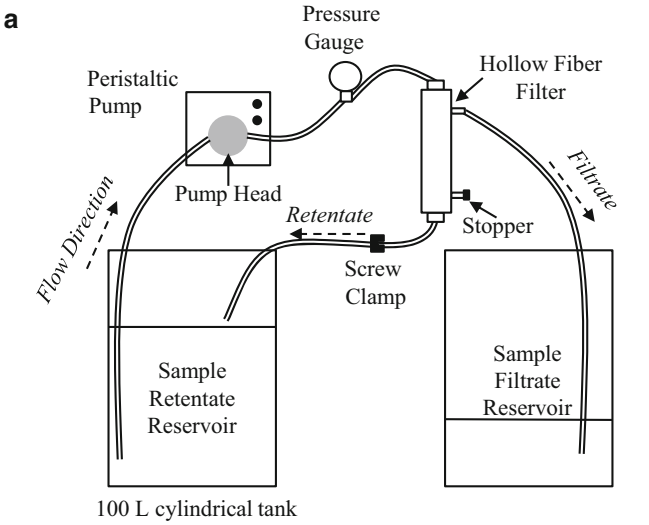
waters. There was a decrease in virus recovery with increasing turbidity levels; however, the recovery rates were still deemed acceptable (Table 10.4). Aside from differences in filtration configurations, DE-HFUF differs from TF-HFUF in the back flushing step. For DE-HFUF, an elution buffer is passed back through the permeate/filtrate port to recover viruses as opposed to recirculation of an elution buffer as done in TF-HFUF. The configuration for DE-HFUF based on Smith and Hill (2009) is shown in Fig. 10.5b and the basic steps are outlined in Fig. 10.4.

2.3. Secondary Concentration

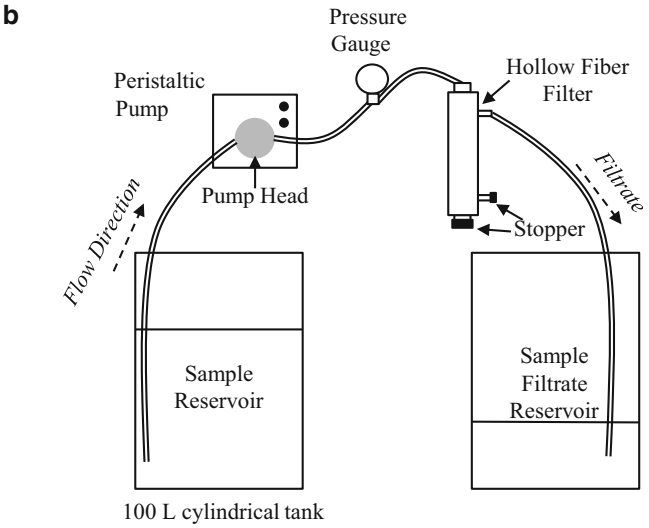
Even though HFUF and VIRADEL methods can concentrate viruses from water by 200- and >4000-fold, respectively, downstream methods for detection of viruses often require a secondary concentration and purification step after the initial concentration step. This additional step is needed since organic compounds (mostly humic acids) and metallic ions are often adsorbed or concentrated along with the viruses during primary concentration. These compounds can have inhibitory effects on downstream detection methods such as RT-PCR and real time qPCR. The methods most commonly used for secondary concentration and purification can be roughly categorized into one of the following groups: flocculation (organic or chemical), polymer-based hydroextraction, and centrifugal ultrafiltration.

One common method for secondary concentration is organic flocculation of the filter eluate (eluent + viruses) obtained during VIRADEL methods. This concept was originally proposed by Katzenelson et al. (1976) and involves the acidification of the eluate to pH 3.5 to cause flocculation of proteins, thus trapping any viruses present. The suspended floc is then centrifuged to a pellet followed by resuspension in 30 ml of sodium phosphate buffer. Organic flocculation is the method currently prescribed by USEPA Method 1615 for the concentration and processing of waterborne viruses (Fout et al. 2010). Additional flocculation methods include lanthanum-based chemical flocculation (Zhang et al. 2013) and celite (diatomaceous earth) concentration (Dahling and Wright 1986; Fout et al. 2003; McMinn et al. 2012, 2013).

Other effective methods for secondary concentration of viruses from water include polyethylene glycol (PEG) hydroextraction (Ramia and Sattar 1979; Lewis and Metcalf 1988; Schwab et al. 1996; Huang et al. 2000; Lambertini et al. 2008; Lee et al. 2011) and centrifugal ultrafiltration devices (e.g., Centricon Plus-70) (Hill et al. 2007; Gibson and Schwab 2011; Ikner et al. 2011). Hydroextraction with PEG has been paired with both VIRADEL and HFUF primary concentration methods, whereas centrifugal filtration devices have primarily been used in conjunction with HFUF. As with the primary methods for recovery of viruses, all secondary concentration steps have both advantages and disadvantages including cost, reproducibility, and ease of use, etc.



[Adapted from Hill *et al.* (2007) and USEPA & CDC, 2011]



[Adapted from Smith & Hill, 2009]

Fig. 10.5 Basic setup for recovery of viruses from water using hollow fiber ultrafiltration (HFUF). (**a**) Tangential flow HFUF (TF-HFUF) (b) Dead-end HFUF (DE-HFUF).

Table 10.4 Virus recovery efficiencies[a] for hollow fiber ultrafiltration methods by water type

Reference	*Flow setup*	*Water type*	*Volume (L)*	*Virus type*	*% Recovery*
Olszewski et al. (2005)	DE	G	100	PP7	70
				T1	71
				Poliovirus type 2	82
		S	100	PP7	86
				T1	70
				Poliovirus type 2	69
Hill et al. (2007)	TF	D	100	MS2	97
				ϕX174	71
Smith and Hill (2009)	DE	S[b]	100	MS2	73
Rhodes et al. (2011)	TF[c]	D	100	Poliovirus type 2	104
				MS2	99
Gibson and Schwab (2011)	TF	D	100	Murine NoV type 1	74
				MS2	48
				PRD1	57
		S	100	Murine NoV type 1	41
				Poliovirus type 2	28
				MS2	66
				PRD1	62
Liu et al. (2012)	TF	Reclaim	100	MS2	95[d]
				ϕX174	90[d]
Mull and Hill (2012)	DE	S[e]	100	MS2	66

DE dead-end, *TF* tangential flow; *S* surface water, *G* groundwater, *D* drinking water, *Reclaim* treated, reclaimed wastewater; *NoV* norovirus

[a]Recovery percentages listed are based on primary concentration followed by detection of viruses by either culture-based or molecular-based methods

[b]Tap water amended with SW to achieve 5 NTU

[c]Results from low rate filtration (<1900 ml/min) are reported here

[d]Combined high and low seed recovery efficiency

[e]Average over low (16 NTU), medium (46 NTU), and high (92 NTU) turbidity levels; however, recovery efficiency did decrease with increasing turbidity

3. METHOD SELECTION: WHAT IS IMPORTANT?

Despite differences in the details of elution and secondary concentration steps for various virus filters, the costs for reagents and disposables are about the same; approximately $5.00–10.00 per sample with the exception of secondary concentration by centrifugal UF devices which adds $30 or more per sample. Processing times, however, can differ greatly among the elution and

secondary concentration procedures and depend also on the laboratory equipment and personnel available for processing filters in batches. Time necessary for clean-up and equipment sterilization must also be considered.

What, then, are the criteria for selecting a filtration method for virus sampling? Several criteria are important. (1) If in addition to viruses, concentrating bacteria and protozoa is required, ultrafiltration and glass wool filtration are good choices because there is sufficient evidence to indicate that these methods effectively concentrate a variety of microbial targets. (2) Sample flow rate can be important when there are many sample sites, long travel times between sites, and limited field sampling personnel. The Nanoceram® filter at flow rate of 10 L/min is advantageous in that respect, requiring one third less time than DE-HFUF at 2.9 L/min. (3) For water samples with pH exceeding 7.5, if the standard approach of decreasing pH inline during sampling is not workable, glass wool filtration should be avoided. However, Nanoceram® filters are reported to be effective at pH values of up to pH 9.0. (4) Tangential flow ultrafiltration is not always practical in field settings, although the requirement for large sample volumes to be transported to the laboratory, might also be impractical. (5) DE-HFUF is prone to clogging and virus recovery has been shown to decrease with increases in turbidity. (6) Lastly, intangible factors such as experience and confidence with equipment and technical procedures should be considered when making a decision on selection of virus filter.

Regardless of filtration method, recovery controls are essential to demonstrate the chosen method does, in fact, concentrate the target microbes from the water being sampled. The general approach is to transport a test water volume (e.g., 10–20 L) to the laboratory, seed it with quantified target, filter and process the water, quantify the target in the final concentrate, and calculate the percent of target recovered. Another approach, more representative of field settings and typical for large sample volumes (hundreds of liters), is to filter the sample at the field site with a target volume minus 10 L, transport the remaining 10 L back to the laboratory, seed it with the target microbes, and finish filtration with the same filter from the field (Fout et al. 2010). A companion volume of unseeded test water should also be filtered in order to quantify indigenous target microbes present in the water. This quantity is then subtracted from the numerator of the recovery calculation.

In addition, when recovery is measured by qPCR, a third companion volume of unseeded test water should be filtered and processed identically as the seeded test water. The target microbial seed is then added to the final concentrate created from this third volume and quantified by qPCR. This quantified value is used as the denominator for calculating virus recovery. This step is crucial for accuracy because filtration and the downstream processing steps can affect qPCR inhibition or other water constituents that shift quantification cycle (C_q) values (Borchardt et al. 2013). Lambertini et al. (2008) observed that inhibition during qPCR was detected in the beef extract eluent from the sample spiked with microbes processed by glass wool filtration; however, qPCR inhibition was not detected in the mock beef extract eluent spiked with

microbes indicating that constituents in the water concentrated during filtration contained inhibitors. In this instance, qPCR inhibition underestimated the quantity of microorganisms spiked in the water sample, resulting in an overestimate of virus recovery. Using six viruses, Calgua et al. (2013) demonstrated that percent recoveries varied depending on which step, and consequently which matrix, in the concentration process the viral seeds were quantified.

Ideally, a recovery control would be performed for each sample, but that is impractical in terms of cost and time. Water matrix type (i.e., the combined attributes of turbidity, pH, dissolved organic matter, inorganic compounds, divalent cations, etc.) is an important determinant of recovery for both VIRADEL and HFUF methods (Hill et al. 2007; Lambertini et al. 2008; Mull and Hill 2012; Wu et al. 2013). Insofar as the waters to be sampled are known to vary in their matrices, for example by location, season, or aquifer type, the number of recovery controls can be planned to correspond with these various matrices. However, this number of recovery controls can be impractical as well and ultimately the number of controls is decided in the context of the sampling plan goals and whether potentially false-negative samples can be tolerated. At a minimum, several recovery controls should be performed during the sampling period just to ensure the procedures in the laboratory are working, particularly if there is a change in reagents. For example, beef extract lots are reported to contribute to variation in virus recovery rates (Fout et al. 2010). Regardless of the number of recovery controls, these data should be reported along with the virus results for the unknown field samples.

4. ADVANTAGES OF VIRUS SAMPLING

Human enteric viruses are the primary cause of foodborne disease outbreaks, and the food commodity most often implicated is fresh produce (i.e. leafy greens and berries) (Koopmans and Duizer 2004; Scallan et al. 2011). The common assumption is that fresh produce is contaminated with viruses at the point of preparation or service, instead of during production, harvest, and post-harvest steps (Berger et al. 2010; Hall et al. 2012). However, a limited number of studies are available on occurrence of viruses in agricultural water sources (van Zyl et al. 2006; Cheong et al. 2009; Kokkinos et al. 2012; Pachepsky et al. 2011). This knowledge gap stems, in part, from the regulatory emphasis of agricultural water standards that focus on the control and detection of bacteria, not viruses. In the US, the proposed rule for produce safety and standards for the sanitary quality of agricultural water sources is based on *Escherichia coli* density exceedance criteria established by the US EPA for recreational waters (USEPA 2012). These criteria were previously adopted by the California Leafy Greens Marketing Agreement (LGMA) and several other state produce safety programs (LGMA 2013; USFDA 2014).

The variety of agricultural water sources—groundwater wells, ponds, rivers, streams, irrigation ditches, municipal water, reclaimed (treated wastewater)

water, and irrigated liquid manure, combined with the diversity of potential fecal contamination sources, makes it difficult to rely on a single indicator, like *E. coli*, to reflect the fate, transport, and occurrence of all potential pathogens in agricultural water resources. Several criteria define an effective indicator: (1) rapidly measurable, (2) representative of the pathogens of concern, and (3) fate and transport characteristics similar to pathogens (Brookes et al. 2005). Unfortunately, *E. coli* meets only one criterion, rapid measurement. Rarely is *E. coli* fully concordant with pathogenic virus levels (Payment and Franco 1993; Harwood et al. 2005; Payment and Locas 2011). An *E. coli* positive sample suggests the presence of fecal material and indirectly viruses. However, an *E. coli*-negative sample does not necessarily mean viruses are absent. Sampling for viruses provides the definitive advantage of knowing whether viruses are present without the risk of making incorrect inferences based on *E. coli*.

Virus sampling methods offer several other advantages for ensuring the sanitary quality of foods: (1) Methods capable of filtering large sample volumes (e.g., 1000 L) can help account for any spatial and temporal variability of viruses in the water source; (2) Concentrated samples can be stored frozen and analyzed later if necessary; (3) Continuous sampling instead of grab sampling is possible from low turbidity waters using cartridge filter methods like glass wool or hollow fiber ultrafilters; and (4) Some methods are effective in concentrating multiple classes of pathogens (e.g., protozoa, bacteria, and viruses) in one step.

5. SUMMARY AND CONCLUSIONS

Based on what has been presented here, it is clear that no single method may universally be recognized as superior or suitable for all monitoring scenarios or contamination events. The efficiency, performance consistency, robustness, complexity of the method, and cost are all factors that must be considered when selecting the appropriate method. Additionally, regardless of the method, performance characteristics must be continuously monitored to ensure recovery efficiency and consistency over time. Finally, there is evidence that applying sampling methods targeted for recovery of viruses and other microorganisms would be advantageous (i.e. when compared to traditional 100 ml grab sample methods for detection of fecal indicator bacteria) in the assessment of the sanitary quality of agricultural water sources.

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CHAPTER 11

Molecular Detection Methods of Foodborne Viruses

Preeti Chhabra and Jan Vinjé

1. INTRODUCTION

Foodborne viruses may cause different clinical syndromes, of which hepatitis caused by hepatitis A virus (HAV) and acute gastroenteritis caused by norovirus are the most important based on the number of outbreaks and people affected (Duizer and Koopmans 2009). The incidence of HAV infection varies considerably between developed and developing countries. In developing countries, where the infection is endemic, the majority of persons are infected in early childhood and most adults are immune; hence outbreaks of HAV are rare. In contrast, higher hygiene standards in high resource countries have resulted in a decrease of immunity against HAV and consequently contaminated food is able to more easily cause HAV outbreaks (Duizer and Koopmans 2009). Norovirus is the leading cause of epidemic gastroenteritis in people of all ages and has become the most important cause of pediatric gastroenteritis in countries where rotavirus vaccination has been introduced (Payne et al. 2013). In the US, norovirus has been implicated in 58 % of all foodborne outbreaks with a known etiology (Scallan et al. 2011).

The identification of a viral pathogen as the cause of foodborne illness is typically performed by epidemiologically linking a particular food item that ill patients had consumed with the laboratory detection of a virus in their stool specimens and the absence of the same virus in stool specimens of healthy consumers who did not consumed that particular food. Though most foods are contaminated during preparation and service, contamination may also occur during production and processing, especially in bivalve mollusks and fresh produce (Stals et al. 2013). The detection of viruses in foods poses a greater challenge than most foodborne bacteria because viruses are not able to replicate in food and therefore a pre-enrichment step, as is common for most bacteria, is not possible. In addition, most foodborne viruses cannot be cultured *in-vitro*. For example, there is no robust cell culture system available for norovirus, the most common foodborne virus, although many different cell lines as well as more sophisticated 3D cell culture methods have been tested (Duizer et al. 2004; Takanashi et al. 2014; Papafragkou et al. 2014; Herbst-Kralovetz

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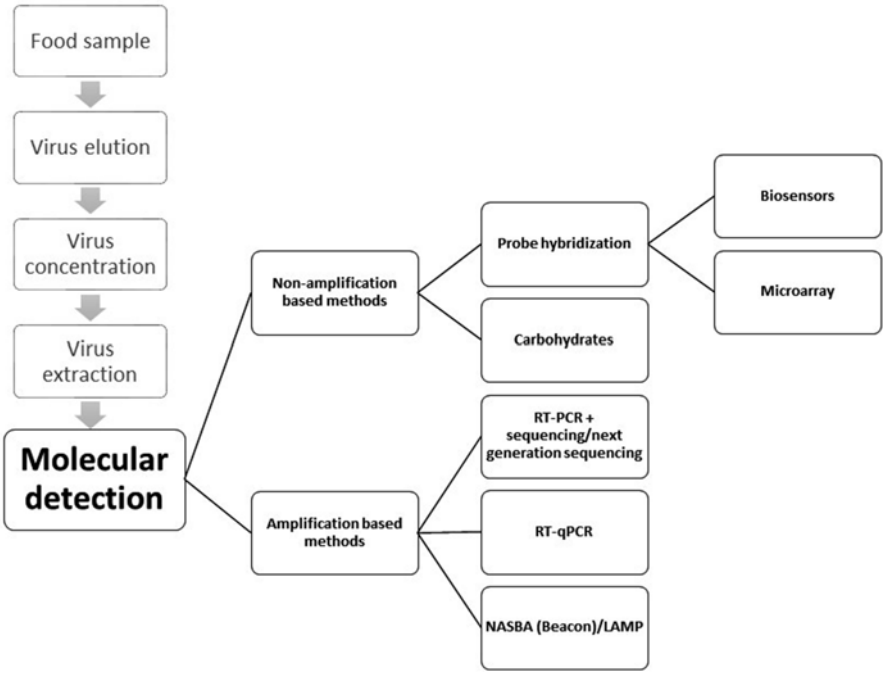

Figure 11.1 Strategies for molecular detection of foodborne viruses.

et al. 2013). Although a recent report indicates that a cell culture system for human noroviruses may not be far away (Jones et al. 2014), the detection of viruses in foods predominantly relies on detection of viral nucleic acid using molecular methods.

The multiple steps for the detection of viruses in foods include: (1) elution of virus from an amount of food (serving size) (2) concentration of virus to a small volume (3) extraction of viral nucleic acid from the concentrated sample, and (4) detection of nucleic acid using molecular methods (Stals et al. 2012) (Fig. 11.1). This chapter provides a review of available molecular detection methods of foodborne viruses from different food matrices.

2. NON-AMPLIFICATION METHODS

2.1. Probe Hybridization

The ability of complementary nucleic acids to form hybrid duplexes has been employed in many molecular techniques, especially for the identification and detection of viral pathogens. Probe hybridization assays were one of the first molecular assays applied to the detection of enteric viruses. In these assays, single-stranded RNA or DNA probes of variable length

(usually 100–1000 bases long) and complementary to a viral genomic sequence are linked to a reporter (radioisotope, enzyme, or chemiluminescent agent) and then hybridized with the ssDNA (Southern blotting) or RNA (Northern blotting) target immobilized on a membrane or *in situ*. Detection of signal from the reporter after the hybridization reaction indicates the presence of the target nucleic acid.

Several different hybridization formats can be used, including solid-phase hybridization, liquid hybridization, and *in situ* hybridization. In solid-phase hybridization, the target nucleic acid is fixed to a nylon or nitrocellulose membrane and a solution containing the labeled probe is applied. After the hybridization reaction, the bound probe is detected by fluorescence, radioactivity, or colorimetric development. In liquid-phase hybridization, both the target and probe are in solution at the time of hybridization. The probe signal can then be detected by measuring fluorescence or color change. Since non-radioactive probe hybridization assays have a detection limit of approximately 10,000 genomic copies they never became the methods of choice for detection of foodborne viruses in foods.

Recent advances in the use of broadly-reactive, virus-specific capture antibodies coupled with various molecular techniques have paved the way towards development of less technically challenging and more rapid pathogen detection methods. Some examples of ligands that can be used to pull viruses out of a complex food matrix are biosensors, nucleic acid aptamers and histo-blood group antigens (HBGA).

2.1.1. Biosensors

Biosensors are potential attractive alternatives to the existing antibody-based detection platforms as they measure a signal released after specific binding of a ligand, such as an antibody, with a pathogen (Fig. 11.2a). A typical biosensor consist of three components: (1) a sensor platform functionalized with a bioprobe, (2) a transduction platform that generates a measurable signal when the analyte is captured and (3) an amplifier component which amplifies and processes the signal to give a quantitative estimate of the analyte (Singh et al. 2013). Biosensors can be directly applied for the detection of a pathogen in food samples generated by mincing and homogenization in the presence of detergents and/or proteolytic enzymes. The choice of sample processing method depends on the type and complexity of the food sample (Singh et al. 2013). Although the use of biosensors for monitoring food and water samples has not been commercialized yet, several recent reports show tremendous potential for this technique (Yakes et al. 2013; Hong et al. 2015).

An electrochemical biosensor using concanavalin A (ConA) as the capture agent was used for the detection of norovirus in optically dense or turbid food extracts (Hong et al. 2015). In this study, a nanostructured gold electrode, which had been treated with ConA and mercaptohexanol, was incubated with different concentrations of norovirus GII.4 seeded in lettuce. The electrode

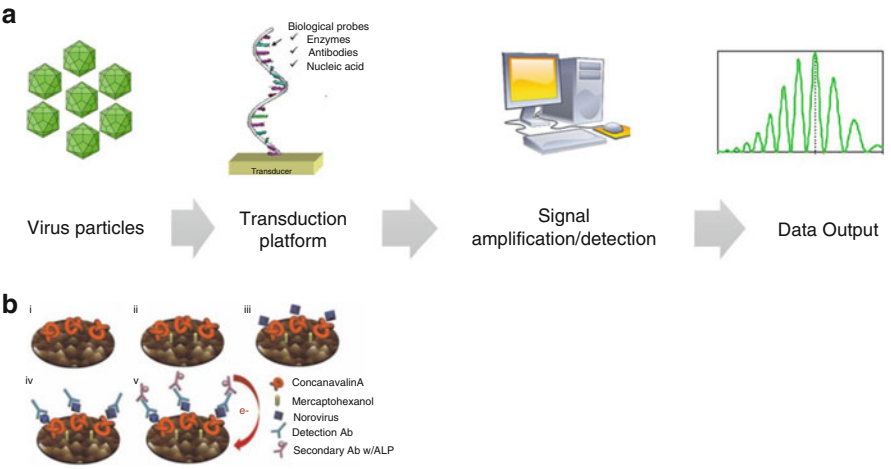

Figure 11.2 A schematic representation of (**a**) various components of a typical biosensor (**b**) electrochemical sensor for norovirus detection (Hong et al. 2015).

was subsequently immersed in a solution of primary and secondary antibodies followed by electrochemical measurement for norovirus sensing (Fig. 11.2b). The system was able to detect norovirus in a concentration range of 10^2–10^6 copies/mL (60 copies/mL as limit of detection). The per sample cost of this method is about 2 % of the typical cost when using an antibody assay and the detection takes not more than 1 h (Hong et al. 2015).

2.1.2. Nucleic Acid Aptamers (Apta-Sensors)

Aptamers are synthetic nucleic acids that fold into unique three-dimensional conformations capable of binding a target with remarkable affinity and specificity (Giamberardino et al. 2013). Nucleic acid aptamers are engineered through repeated rounds of *in vitro* selection, referred to as SELEX (systematic evolution of ligands by exponential enrichment) (Fig. 11.3). Aptamers can bind to various molecular targets such as proteins, nucleic acids, and even cells and tissues and have been employed successfully in the detection of foodborne pathogens including HAV and norovirus (Jaykus and Brehm-Stecher 2013; Escudero-Abarca et al. 2014; Heiat et al. 2014). This technology uses a RNA or DNA aptamer that is immobilized on microtiter plates and virus is detected using an enzyme-labeled monoclonal antibody. Hwang et al. (2007) were able to detect 100 pg/mL of hepatitis C virus and 180 viral particles of murine norovirus (Hwang et al. 2007). A dilution series of GII.4 norovirus was used to artificially contaminate lettuce samples and the virus was recovered using a combined pre-concentration-Aptamer Magnetic Capture (AMC)-RT-qPCR assay; as few as 10 RNA copies could be detected with a capture efficiency of 36 % (Escudero-Abarca et al. 2014). These data are very

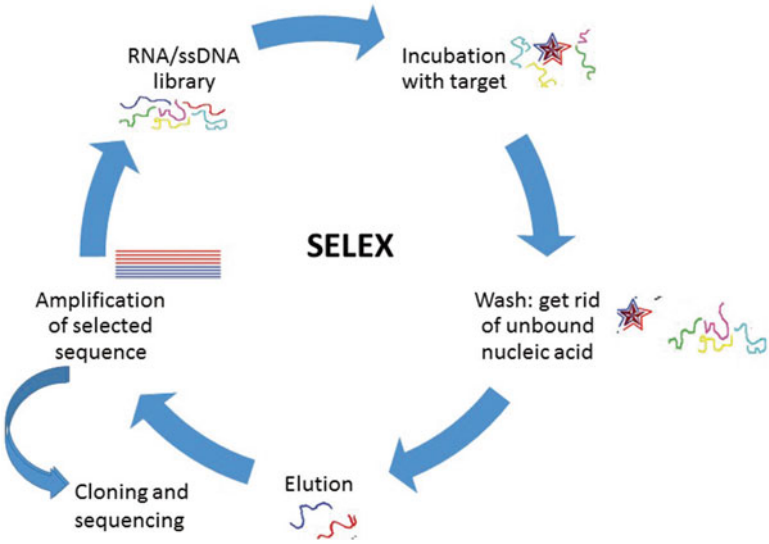

Figure 11.3 A schematic depiction of the systematic evolution of ligands by exponential enrichment (SELEX) technique for aptamer selection.

promising and should be repeated for different norovirus genotypes in different food items as well as on epidemiologically implicated naturally contaminated food items.

2.1.3. Histo-Blood Group Antigens

Interactions of carbohydrates with proteins or other carbohydrates play a key role in binding, entry and intracellular processes after an encounter with a pathogen. The involvement of carbohydrate moieties in norovirus binding to human gastrointestinal cells has been well documented (Marionneau et al. 2002; Chakravarty et al. 2005; Tan and Jiang 2005; Hutson et al. 2004). Histo-blood group antigens (HBGAs) are complex carbohydrates linked to glycoproteins or glycolipids that are present as free antigens on various biological surfaces derived from plants and animals and can serve as binding ligands to capture noroviruses (Esseili et al. 2012; Tian et al. 2006; Tian et al. 2007). These carbohydrates have been used successfully to capture and detect noroviruses on lettuce, clams, mussels and oysters (Esseili et al. 2012; Tian et al. 2006; Tian et al. 2007; Le Guyader et al. 2006). Oyster species express type A– and type O–like HBGA structures on their gastrointestinal tissue (Tian et al. 2007). The terminal N-acetylgalactosamine residues of type A HBGAs serve as candidate receptors for norovirus and have been shown to trap GI and GII viruses as well as recombinant virus-like particles (Hutson et al. 2004). Type A-like HBGA was also found in mussels and clams (Tian et al. 2007). Cell wall structures on lettuce are able to bind virus like particles, which if confirmed

for native virions, may imply that noroviruses could persist on lettuce leaves (Esseili et al. 2012).

2.1.4. Quantum Dots (Nanosensors)

Luminescent semiconductor nanocrystals or quantum dots (QDs) have unique optical properties that are advantageous for the development of novel chemical sensors and biosensors. The QDs are powerful fluorescent probes that have several advantages over organic dyes which include higher brightness, better resistance to photobleaching, and simplified multicolor target detection. These properties enable the use of QDs as optical labels for faster and easier molecular detection of biological targets. By using antibodies raised against a panel of different pathogens, QDs have the potential to simultaneously detect multiple viruses using the fluorescence intensity of the multicolored QDs, which are excited at the same single wavelength and emit different colors (Lee et al. 2013). The use of QDs for the qualitative detection of norovirus from fresh lettuce was recently reported; it allowed the detection of norovirus from a food samples within 2 h (Lee et al. 2013). Combining QDs with immunomagnetic separation has the potential to simultaneously detect several different foodborne viruses.

2.1.5. Microarrays

The core principle behind microarray technology is hybridization between two complementary DNA strands. Typically, hundreds and/or thousands of samples are immobilized on a solid support (microscope glass slides, silicon chips or nylon membranes). The size of the sample spot is typically less than 200 μm in diameter. The spots can be DNA, cDNA, or oligonucleotides (Fig. 11.4). To generate the labeled ssDNA template, viral RNA is first converted into complementary DNA (cDNA) which itself can be fluorescently labelled (e.g., Cy3 and Cy5) or can serve as a template to generate PCR products followed by post-PCR fluorescent labelling. The fluorescently labelled cDNA or PCR products are then denatured to obtain labelled ssDNA. Ideally, each molecule of the labelled ssDNA will only bind to its appropriate complementary target sequence spotted on a solid support matrix and generate a signal. The total strength of the signal is directly correlated with how many molecules of the sample nucleic acid are bound to the probe. A laser excites each spot and the fluorescent emission is gathered through a photo-multiplier.

Initial attempts to develop microarray assays to detect and/or genotype enteric viruses were limited to detection of known mutations in the viral genome based on hybridization to a "handful" of oligonucleotide probes immobilized on a solid support (Nolte and Caliendo 2003); Chizhikov et al. 2002; Lovmar et al. 2003). Reverse line blot arrays, or microarrays, were one of those beginning assays that allowed the use of multiple oligonucleotide probes on a solid matrix (e.g. nylon membrane). In this method, one of the oligonucle-

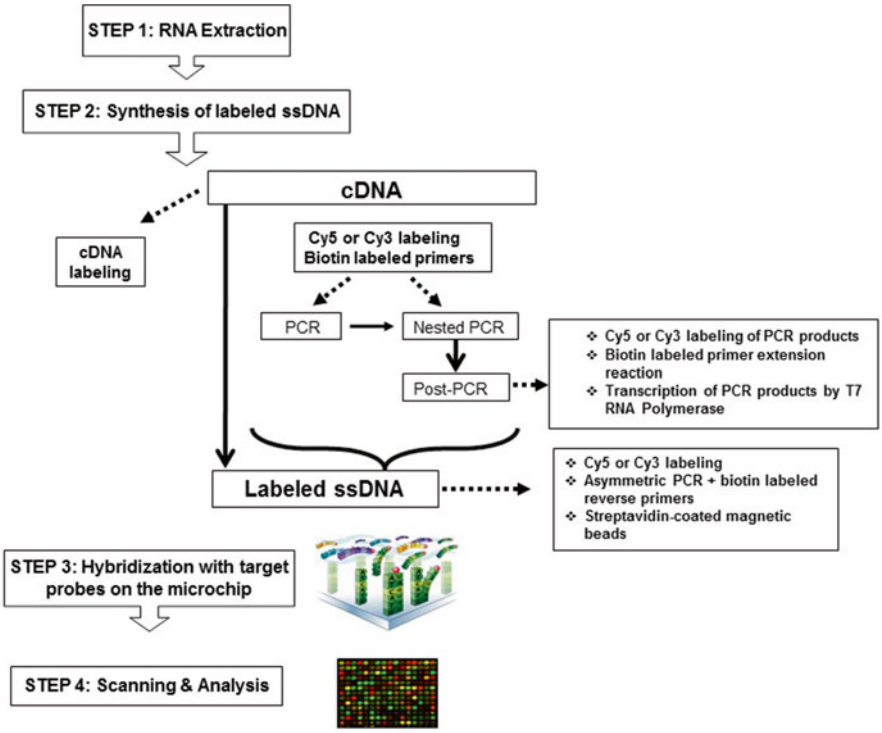

Figure 11.4 Work flow for detection and genotyping of foodborne viruses by microarray.

otide primers used during PCR amplification is biotinylated and hybridization of the DNA strand containing the biotinylated primer to a virus-specific probe bound to a solid matrix is detected by a peroxidase-labelled streptavidin reporter. This method has been used successfully to identify and characterize norovirus strains using genotype-specific probes (Vinje and Koopmans 2000). This technology was further adapted for high-throughput analysis with the use of DNA microarrays (Nolte and Caliendo 2003; Chizhikov et al. 2002; Lovmar et al. 2003).

Microarrays have been used in combination with RT-PCR for the detection and genotyping of group A rotaviruses (Chizhikov et al. 2002; Lovmar et al. 2003) and norovirus (Pagotto et al. 2008; Mattison et al. 2011). The norovirus microarray (NoroChip v3.0) has been developed for simultaneous detection and genotyping of GI and GII noroviruses. Probes were selected targeting a partial capsid region (region C) and a 2.4 kb amplicon spanning the polymerase region, and capsid sequences were to be amplified from each sample. The array provides an oligonucleotide hybridization platform to screen over 600 potential interactions in each experiment (Mattison et al. 2011). Several high-throughput, high-density microarrays for the simultaneous detection of

norovirus, HAV, rotavirus, adenovirus, astrovirus, and coxsackie viruses A and B have been described (Kostrzynska and Bachand 2006; Jaaskelainen and Maunula 2006; Ayodeji et al. 2009; Chen et al. 2011; Kim et al. 2012). However, these assays have only been used for the detection of viruses grown in cell cultures or in stool samples of patients. Direct detection of foodborne viruses in food matrices using microarrays has not been reported yet.

3. TARGET SPECIFIC AMPLIFICATION METHODS

To date, most molecular methods to detect foodborne viruses are based on polymerase chain reaction (PCR) amplification of a partial region of the viral genome. Since the initial description of the PCR assay (Saiki et al. 1985), PCR and real time PCR (qPCR) methods have become the gold standard for detection of foodborne viruses and have been successfully employed for the detection of norovirus and HAV in naturally contaminated foods. Various combinations of oligonucleotide primers and probes for the semi-quantitative detection of norovirus and HAV in food have been reported (Table 11.1). Standardized method have been developed for detecting both of these viruses (TAG 4 of CEN/TC 275/WG 6) and have been approved (CEN ISO/TS 15216-parts 1 & 2) (Hennechart-Collette et al. 2015; Standardization 2013a; Standardization 2013b). This is an excellent example of how to best harmonize the optimum molecular approach based on consensus among many different European research groups and this CEN method should be considered by other countries including the US.

3.1. Conventional Polymerase Chain Reaction (PCR) Formats for Detection of Foodborne Viruses

Polymerase chain reaction (PCR) is a method to amplify a specific DNA sequence generating thousands to millions of copies of that sequence. Typically, the target DNA sequence is amplified using a solution containing DNA polymerase, deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP), and target-specific oligonucleotide primers that flank the DNA region to be amplified and are complementary to sequences on opposite strands of the target DNA sequence. An appropriate buffer is also included for optimum activity and stability of the DNA polymerase. There are three basic steps that are repeated through a variable number of cycles: (1) heat denaturation, (2) primer annealing, and (3) primer extension. During heat-denaturation, double-stranded DNA (dsDNA) will denature into two separate strands. The reaction mixtures are then cooled to a temperature that allows the primers to anneal to the target sequences of the separated DNA strands. During the primer extension step, the DNA polymerase then forms a new strand by extending the bound primers with nucleotides, creating a complimentary copy of the target DNA sequence (Fig. 11.5). When repeated, this cycle of denaturing,

Table 11.1 Commonly used oligonucleotide primers and probes for TaqMan real time RT-PCR detection of GI and GII norovirus and hepatitis A virus in food matrices

Virus	*Target region*	*Name*	*DNA sequence (5'–3')*	*Reference*
GI norovirus	ORF1-2 junction	JJV1F	GCC ATG TTC CGI TGG ATG	Jothikumar et al. (2005a, b)
		JJV1R	TCC TTA GAC GCC ATC ATC AT	
		JJV1P	**FAM**-TGT GGA CAG GAG ATC GCA ATC TC-**BHQ**	
GII norovirus	ORF1-2 junction	JJV2F	CAA GAG TCA ATG TTT AGG TGG ATG AG	
		COG2R	TCG ACG CCA TCT TCA TTC ACA	
		RING2-TP	**FAM**-TGG GAG GGC GAT CGC AAT CT-**BHQ**	
GI norovirus	ORF1-2 junction	COG1F	CGY TGG ATG CGN TTY CAT GA	Kageyama et al. (2003)
		COG1R	CTT AGA CGC CAT CAT CAT TYA C	
		RING1(a)-TP	**FAM**-AGA TYG CGA TCY CCT GTC CA-**TAMRA**	
		RING1(b)-TP	**FAM**-AGA TCG CGG TCT CCT GTC CA-**TAMRA**	
GII norovirus	ORF1-2 junction	COG2F	CAR GAR BCN ATG TTY AGR TGG ATG AG	
		COG2R	TCG ACG CCA TCT TCA TTC ACA[a]	
		RING2-TP	**FAM**-TGG GAG GGC GAT CGC AAT CT-**TAMRA**	
GII norovirus	ORF1-2 junction	QNIF2d (+)	ATG TTC AGR TGG ATG AGR TTC TCW GA[a]	Loisy et al. (2005)
		QNIFS (+)	**FAM**-AGC ACG TGG GAG GGC GAT CG-**TAMRA**[a]	
GI norovirus	Capsid	NV1LCF	CAR GCC ATG TTY CGY TGG ATG	Svraka et al. (2007)
		NV1LCR	CCT TAG ACG CCA TCA TCA TTT AC[a]	
		NV1LCpr	**VIC/FAM**-TGG ACA GGA GAY CGC RAT CT-**TAMRA**[a]	
GII norovirus	Capsid	NV2LCF	GAR YCI ATG TTY AGR TGG ATG	
		NV2LCR	TCG ACG CCA TCT TCA TTC AC	
		NV2LCpr	**FAM**-TGG GAG GGS GAT CGC RAT CT-**TAMRA**	
GI norovirus	Capsid	QNIF4	CGCTGGATGCGNTTCCAT[a]	da Silva et al. (2007)

(continued)

Table 11.1 (continued)

Virus	*Target region*	*Name*	*DNA sequence (5′–3′)*	*Reference*
Hepatitis A virus	5′ UTR	HAV-for	GGG TAA CAG CGG CGG ATA T	Hewitt and Greening (2004)
		HAV-rev	TTA AGC CTA AAG ACA GCC CCG	
		HAV	**FAM**-TCA ACG CCG GAG GAC TGA CTC TCA TC-**BHQ-1**	
Hepatitis A virus	5′ UTR	Forward primer	GGT AGG CTA CGG GTG AAC	Jothikumar et al. (2005a, b)
		Reverse primer	GCG GAT ATT GGT GAG TTG TT	
		Probe	**FAM**-CTT AGG CTA ATA CTT CTA TGA AGA GAT GC-**BHQ**	
Hepatitis A virus	5′ UTR	HAV68	TCA CCG CCG TTT GCC TAG[a]	Costafreda et al. (2006)
		HAV240	GGA GAG CCC TGG AAG AAA G[a]	
		HAV150	**FAM**-CCT GAA CCT GCA GGA ATT AA-**MGB-NFQ**[a]	
Hepatitis A virus	5′ UTR	Forward primer	AGG CTA CGG GTG AAA CCT CTT AG	Hu and Arsov (2009)
		Reverse primer	CGC CGC TGT TAC CCT ATC C	
		Probe	**FAM**′-AAT ACT TCT ATG AAG AGA TGC C-**BHQ**	
Hepatitis A virus	5′ UTR	Primer forward	GCG GCG GAT ATT GGT GAG	Di Pasquale et al. (2010)
		Primer reverse	CAA TGC ATC CAC TGG ATG AGA	
		Probe	**FAM**-TTA AGA CAA AAA CCA TTC AAC GCC GGA G-**TAMRA**	

UTR untranslated region, *FAM* 6-carboxyfluorescein, *TAMRA* tetramethylrhodamine, *VIC* 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein, *BHQ* black hole quencher, *MGB-NFQ* minor groove binder-nonfluorescent quencher

[a]Primers and probes used for detection of hepatitis A virus and GI and GII noroviruses present in food matrix by CEN ISO/TS 15216-parts 1 & 2 (Standardization 2013a; Standardization 2013b)

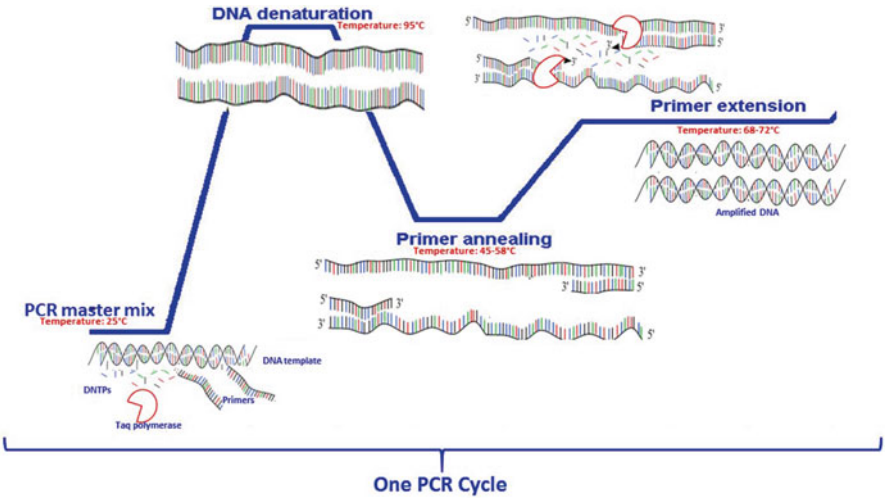

Figure 11.5 Schematic of the polymerase chain reaction.

annealing, and extending increases the number of target DNA sequences exponentially. Ideally, no amplification should occur if the target DNA sequence is not present.

Reverse transcription-PCR (RT-PCR), also called RNA-PCR, is a modification of the PCR reaction that allows amplification of an RNA template. In the initial step, complementary DNA (cDNA) is synthesized, which is then amplified by normal PCR using cDNA as the template. The cDNA synthesis step also requires deoxyribonucleotide triphosphates, an oligonucleotide primer, an appropriate buffer and a DNA polymerase with reverse transcriptase activity. The oligonucleotide primer can be template-specific (as used in the PCR reaction), random hexamers, or oligo-dT (if the genomic region to be amplified is near the polyadenylated 5′-end of the genome, such as norovirus and HAV). In a two-step RT-PCR assay, the cDNA is synthesized in a separate reaction and all or a part of the reaction mix is subsequently added to the PCR reaction mix. In a one-step RT-PCR assay, all reagents necessary for both cDNA synthesis and PCR amplification are added at the same time.

Nested and/or hemi-nested PCR is the serial amplification of a target sequence using two different oligonucleotide primer pairs of which the second set is internal (nested) to the first set (Haqqi et al. 1988). By definition, nested PCR also increases the specificity of the assay because both primer pairs must amplify the target sequence. The initial amplification is performed using an outer primer pair and 20–30 cycles of amplification. A second round of amplification is then performed using primers that anneal to a region internal (nested) to the initial two primers. Hemi-nested PCR is a variant of nested PCR in which one of the primers used in the second round of amplification is the same as that used in the first round of amplification and the second primer

anneals to a region on the opposite strand that is nested between the initial two primers. Nested and hemi-nested PCR have been used to detect sequences that cannot be detected after a single round of PCR thus increasing the sensitivity of the assay.

Multiplex PCR assays use two or more primer pairs to amplify different target sequences in a single tube (Chamberlain et al. 1988). This strategy allows for the evaluation of a sample for more than one virus at a time or multiple genotypes of the test virus at the same time. However, the primers for different targets should have similar annealing temperatures and lack complementarity so that each target can be efficiently amplified.

3.1.1. Post Amplification Analysis and Interpretation of Results of Conventional PCRs

In order to interpret conventional RT-PCR results, the products of appropriate size must be confirmed by DNA sequencing, restriction analysis, or hybridization assays. Direct DNA sequencing of PCR amplicons (Sanger sequencing) is the most common approach used these days in most laboratories to identify and confirm a positive PCR result. Sequence data not only confirms the specificity of the PCR amplification but also been directly used for genotyping or classification of virus strains (Robertson et al. 1991; Vega et al. 2014). Combined with epidemiologic data, genotyping information has become a worldwide accepted tool for surveillance and outbreak investigations (Koopmans et al. 2003). The dye-terminator variant of Sanger sequencing is used by most of the commercially available automated DNA sequencers. Because of the increased availability of these sequencers this has become the method of choice for routine laboratory testing. Sanger sequencing is reliable for sequencing amplicons up to 1000 base pairs in length.

Current molecular typing tools based on conventional PCR amplification followed by Sanger sequencing lack the resolution for differentiating tightly linked viral strains and do not differentiate certain viral strains and/or viral quasispecies. Also, Sanger sequencing is limited to sequences up to 1000 nucleotides However, advances in sequencing technology to overcome the limitations of Sanger sequencing have led to the development of various high-throughput next-generation sequencing (HT-NGS) platforms such as sequencing-by-synthesis 454 Life Sciences, Illumina (Solexa) sequencing, SOLiD sequencing, and the Ion Torrent semiconductor sequencing technologies, all of which use different detection principles (Diaz-Sanchez et al. 2013). More recently, third generation sequencing technologies have been developed which include Nanopore Sequencing, single molecule real-time sequencing, and multiplex polony technology (Kilianski et al. 2015).

Massively parallel DNA sequencing systems which can determine the sequence of large numbers of different DNA strands at a time are now available in the commercial marketplace. These systems allow millions of “reads” (con-

tiguous regions of DNA sequence) to be gathered in a single experiment, in contrast to current capillary electrophoresis instruments (Hert et al. 2008). The next generation sequencing technologies can be employed in various different ways to provide accurate subtyping and cluster analysis for investigating foodborne outbreaks and trace back to the food or environmental source. Hybridization is another common approach used to confirm and type positive PCR results. This approach is discussed in detail in Sect. 2.1 above. Restriction digest of virus-specific amplicons using a restriction endonuclease combined with separation of the digested (shorter) products by gel electrophoresis is an alternative, but less common technique for confirmation of PCR results, due to the continued lower cost of sequencing.

3.2. Real-Time PCR

The key feature of a real-time PCR is that the amplified DNA is detected as the reaction progresses in "real time", compared to conventional PCR with its end-point detection. The beauty of real-time PCR is that it is able to improve the efficiency of the analytic process while decreasing the risk of carryover contamination by eliminating the need for post-PCR analysis. Two common methods for the detection of products in quantitative PCR are: (1) non-specific fluorescent dyes such as SYBR Green that intercalate with any double-stranded DNA and fluoresce after exposure to a specific wavelength, and (2) sequence-specific DNA probes (oligoprobes) consisting of oligonucleotides labelled with a fluorescent reporter at the 5′-end and a quencher at the 3′end which allow detection only after hybridization of the probe with its complementary sequence to quantify the target RNA or DNA.

SYBR Green is the most commonly used non-specific fluorescent dye. Other options are ethidium bromide and YO-PR0-1. SYBR Green-based detection is the least expensive and easiest method available for real-time PCR. Detection of the fluorescent signal occurs during each PCR cycle at the end of either the annealing or the extension step. SYBR Green detects any double-stranded DNA non-specifically and, depending on the level of nonspecific binding to other DNA templates present in the reaction, could potentially produce non-specific products. A melting curve analysis should be considered after the SYBR Green PCR to determine if the product is specific. However, this limits the ability of this approach to identify amplicons especially when the target is present in low concentrations as can be expected in contaminated foods (Mackay et al. 2002).

TaqMan probes are the most commonly used fluorescently-labeled oligoprobes. They are hydrolysis probes that are designed to increase the specificity of quantitative PCR. These probes contain a fluorescent reporter dye at the 5′ end of the probe and a quencher dye at the 3′ end of the probe. When the probe is intact, the proximity of the reporter dye to the quencher dye suppresses the reporter fluorescence (Fig. 11.6). Probe cleavage during the PCR reaction spatially separates the reporter dye from the quencher dye, thereby

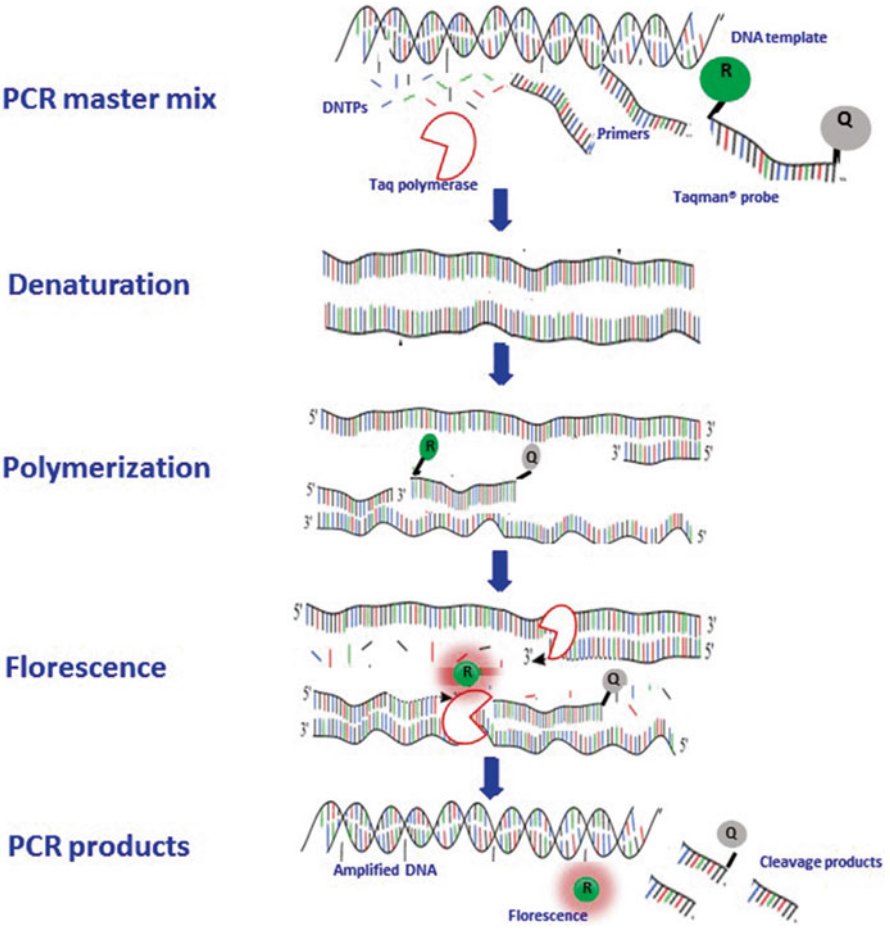

Figure 11.6 Principles and chemistry of the TaqMan-based real-time PCR assay.

allowing detection of the reporter dye fluorescence (Holland et al. 1991). RNA is first reverse-transcribed to cDNA and then subjected to standard PCR. If the target DNA is amplified, the TaqMan probe hybridizes to the sequence. The 5′–3′ nuclease activity of the DNA polymerase cleaves the hybridized fluorogenic probe and the fragments of the reporter dye are displaced from the target, resulting in an increase in fluorescence. This step occurs in all amplification cycles but does not interfere with the exponential accumulation of product during PCR. The 3′ end of the probe is blocked by the quencher dye to prevent extension of the probe during PCR. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. The increase in fluorescence occurs only if the target sequence is comple-

mentary to the probe and is amplified during PCR. Nonspecific amplification is not detected in the absence of a probe-binding site. The 5′ nuclease assay is specific to a pre-determined target.

3.3. Controls: Process and Amplification Controls

Since PCR is theoretically able to detect a single copy of target sequence, this method can easily lead to cross-contamination. Therefore, appropriate controls (positive, negative, and matrix extraction) are critical to include in each experiment to avoid false-positive results. On the other hand, failure to remove inhibitory substances during nucleic acid extraction and purification steps or the inefficient recovery of virus from a sample can lead to false-negative results. In general, enteric viruses present in food are difficult to detect due to (1) the often relatively low levels of viral contamination, (2) inability of the virus to grow in food and, (3) the presence of substances in the complex food matrices that are difficult to remove during sample processing (Lee et al. 2012; Maunula et al. 2013; Suffredini et al. 2014; Hennechart-Collette et al. 2015). All these factors may inhibit PCR amplification and consequently mask the presence of a viral pathogen (Martin D'Agostino et al. 2011). Therefore, it is essential that when testing foodstuff for viruses, appropriate quality controls are included to verify that the method was performed correctly. All process controls should be added to the food prior to nucleic acid extraction and all amplification controls should be added prior to PCR.

3.3.1. Process Controls

To determine the presence of a foodborne virus, it is critical to include process controls prior to virus extraction to monitor all the analytical steps involved, from sample elution and concentration, extraction of viral nucleic acid, virus detection as well as interpretation of results. Two types of process controls should be added to the food sample: a positive process control (PPC) and a negative process control (NPC). PPC is usually a non-target virus that is added to each test sample including the negative control sample. The selected PPC should have morphological, physicochemical and environmental persistence properties similar to those of the target viruses, thus providing comparable extraction efficiency and/or recovery rates. Also, the PPC virus should be easy to cultivate and to detect, non-pathogenic for humans and should not be a natural contaminant of the tested food sample (Martin D'Agostino et al. 2011). Mengovirus, murine norovirus, feline calicivirus and bacteriophage MS2 have been used successfully as PPC for the detection of hepatitis A and E viruses, noroviruses and other enteric viruses in shellfish and several other food samples (Dreier et al. 2005; Costafreda et al. 2006; Le Guyader et al. 2009; Uhrbrand et al. 2010; Hennechart-Collette et al. 2014; Martin-Latil et al. 2012a, b; Stals et al. 2011b; Martin-Latil et al. 2014; Blaise-Boisseau et al. 2010; Mattison et al. 2009; Hennechart-Collette et al. 2015). An NPC, which is usually sterile

distilled water, is also carried through all steps from sample preparation to virus detection to monitor any cross-contamination events.

3.3.2. Amplification Controls

Amplification controls are added prior to the amplification step to monitor the performance of the (RT)-PCR assays. Examples of different types of amplification controls added before PCR reaction are: internal amplification control (IAC), External amplification control (EAC), positive amplification control (PAC), and negative amplification control (NAC) (Martin D'Agostino et al. 2011). The incorporation of an internal amplification control (IAC) will identify failed reactions. An IAC is a nucleic acid sequence (often, but not always, the process control virus) that is added to each PCR reaction. This control is co-amplified with the target sequence and is distinguishable from the target sequence by size of the PCR product in conventional PCR or through detection by a specific probe in real-time PCR (Rodriguez-Lazaro et al. 2004; Rodriguez-Lazaro et al. 2005; Martin D'Agostino et al. 2011). In a reaction without an IAC, a negative response can mean either that there is no target sequence present in the reaction or that the amplification has failed most likely due to inhibition. The absence of response both from the target and the IAC indicates that the reaction has failed, and the sample must be retested to avoid any false negative interpretation of its analysis. An IAC is required both for the target virus and the Sample Process Control Virus (Martin D'Agostino et al. 2011).

As an alternative to IAC, an EAC can be used to identify possible inhibitors that are co-extracted with the nucleic acid from the sample. An EAC may be identical to the target nucleic acid sequence. Therefore, two separate reactions must be run for each sample. One (the test) reaction contains only the sample nucleic acid, whereas the other (control) reaction contains the sample nucleic acid plus the EAC (Costafreda et al. 2006). If the EAC is successfully amplified (i.e., produces a signal), a negative result is considered to be a true negative, assuming that both amplification reactions performed with similar efficiency. If, however, no signal is produced in both the test and control reactions, the nucleic acid extract contains inhibitory substances and the test reaction has failed (Martin D'Agostino et al. 2011). Other controls include a PAC which is usually added to verify amplification performance of the target sequence. A PAC can be a natural virus or chimerical nucleic acids. When an IAC or EAC is used, there is no need to add PAC. However, PAC is usually used along with IAC or EAC in a reaction involving a probe for the target to verify probe performance (Martin D'Agostino et al. 2011). NAC (or No Template Control-NTC- or Reagent Control or Blank) is used to verify that the (RT-) PCR master mix reagents are not contaminated with target virus or with any of the control and/or their amplicons used in the experiment. Usually, water is added instead of the template. A negative signal indicates

the absence of specific contamination in the amplification assay (Rodriguez-Lazaro et al. 2005; Martin D'Agostino et al. 2011).

3.3.3. Interpretation of PCR and qPCR Results Based on Control Results

Interpretation of qPCR results is important when monitoring and analyzing food samples for the presence of enteric viruses by PCR-based methods. In a valid assay, all process and amplification controls should work as expected (Table 11.2). For example, a sample process control must be detected in every sample and no target virus signal should be present in the negative process control and negative reagent control. A negative signal for an internal amplification control could either mean that the amplification has failed due to the presence of inhibitors or that no target sequence was present in the reaction. The sample must then be retested for correct interpretation of results (Table 11.2).

3.4. Application of Different Conventional and Real-Time PCRs in Detection of Viruses in Food Matrix

During the 1990s conventional RT-PCR assays were employed successfully for the detection of norovirus and HAV in shellfish and on environmental surfaces (Zhou et al. 1991; Atmar et al. 1995; Lees et al. 1995). These methods have also been used successfully for virus detection in naturally contaminated and/or experimentally spiked food such as melons, lettuce, berries, hamburger meat, and sliced deli meats (Gouvea et al. 1994; Bidawid et al. 2000; Leggitt and Jaykus 2000; Schwab et al. 2000; Le Guyader et al. 2004). Some studies have successfully utilized these methods to detect viruses in foods associated with outbreaks of human disease. For example, norovirus was detected in foods such as ham and raspberries that had been implicated epidemiologically as the cause of the outbreak (Schwab et al. 2000; Le Guyader et al. 2004). HAV was detected in blueberries that had caused an outbreak (Calder et al. 2003) and HAV or norovirus has been frequently detected in outbreaks associated with the consumption of shellfish (Jansen et al. 1990; Le Guyader et al. 1996; Le Guyader et al. 2000; Le Guyader et al. 2003; Sugieda et al. 1996; Shieh et al. 1999; Prato et al. 2004; Formiga-Cruz et al. 2002).

In 2003 Kageyama et al. published a seminal paper on the successful development of a norovirus GI and GII real time RT-PCR targeting the most conserved region (ORF1-ORF2 junction) of the norovirus genome (Kageyama et al. 2003). Using the oligonucleotide primers described in this publication, many researchers developed SYBR green based RT-qPCR assays for norovirus (Richards et al. 2004; Schmid et al. 2004; Pang et al. 2004; Laverick et al. 2004). Other SYBR green based assays targeting VP3-VP1 region have also been developed to detect HAV RNA in water and cell culture samples (Brooks et al. 2005; (Gersberg et al. 2006).

Table 11.2 Interpretation of results

Observation	*Results*	*Interpretation of results*
Target virus positive + PPC virus positive + IAC and/or EAC and/or PAC positive + NPC/NAC negative	Nucleic acid extraction and amplification successfully done	Sample positive for target virus
Target virus negative + PPC virus positive + IAC and/or EAC and/or PAC positive + NPC/NAC negative	Nucleic acid extraction and amplification successfully done	Sample negative for target virus
Target virus positive + PPC virus positive + NPC positive + IAC and/or EAC and/or PAC positive + NAC signal negative	Contamination of reagent/equipment with PPC in any step of sample processing	Decontaminate the equipment used and repeat complete method again with new set of reagents
Target virus positive/negative + PPC virus positive + IAC and/or EAC and/or PAC positive + NPC negative + NAC positive	There is a possibility of reagent/equipment contamination with target virus and/or any of the positive controls during amplification step	Decontaminate the equipment used and repeat amplification step again with new set of reagents
Target virus positive + PPC virus negative + IAC and/or EAC and/or PAC positive + NPC/NAC negative	Nucleic acid extraction has failed and there is a possibility of reagent/equipment contamination with target virus OR PPC was not added to the sample in the initial stages OR Titer of PPC stock have reduced	First, check the titer of the PPC stock to rule out the possibility of reduction in PPC stock titers. If stock titers are good then proceed with decontamination of the equipment used and repeat complete method again with new set of reagents

Target virus negative + PPC virus negative + IAC and/or EAC and/or PAC positive + NPC/NAC signal negative	Nucleic acid extraction had failed OR Possibility of inhibition	Dilute nucleic acid to 1:10 and 1:100 and repeat amplification step. If both target virus + PPC are positive in diluted sample and all other controls are correct then consider sample positive for target virus If target virus is negative + PPC is positive in diluted sample and all other controls are correct then consider sample negative for target virus If both target virus + PPC are negative in diluted sample then repeat complete method again
Target virus negative + PPC virus positive + IAC and/or EAC and/or PAC negative +NPC/NAC signal negative	Nucleic acid amplification has failed	Repeat amplification step again
Target virus negative + PPC virus negative + IAC and/or EAC and/or PAC negative + NPC/NAC negative	Nucleic acid extraction and/or amplification has failed	Repeat complete method again

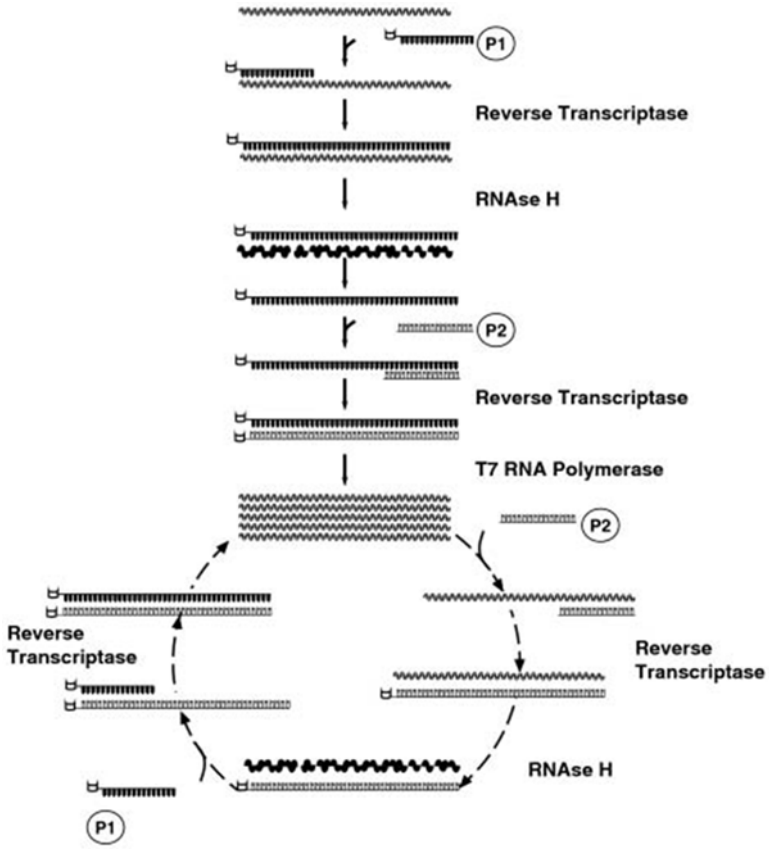

Figure 11.7 Nucleic acid sequence-based amplification (NASBA): a one-step isothermal process for amplifying RNA (Lauri and Mariani 2009).

However a more specific approach is the use of TaqMan RT-qPCR assays which confirm the specific norovirus amplification as well as provides at least semi-quantitative data. Hence, TaqMan RT-qPCR assays are now the most widely used methods for the detection of norovirus and HAV in high-risk foods (Table 11.1). Using RT-qPCR, various food products have been linked to foodborne viral disease outbreaks. Such foods include bivalve mollusks (especially raw or undercooked oysters), raw shellfish, fresh fruit and vegetables including different types of lettuce, onions, soft red fruits, berries, and more recently, semidried tomatoes (Le Guyader et al. 2010; Thebault et al. 2013; Lowther et al. 2010; Bernard et al. 2014; Fournet et al. 2012; Gallot et al. 2011; Sarvikivi et al. 2012; Gallimore et al. 2005; Makary et al. 2009; Wadl et al. 2010; El-Senousy et al. 2013; Baert et al. 2011; Jothikumar et al. 2005a, b; Le Guyader et al. 2009; Boxman et al. 2006; Croci et al. 2007; Nishida et al. 2003; Terio et al. 2010; Le Guyader et al. 2004; Maunula et al. 2009; Costafreda et al. 2006; Butot et al. 2007; Blaise-Boisseau et al. 2010).

3.5. Isothermal Amplification Methods

In contrast to PCR technology, isothermal amplification is carried out at a constant temperature (i.e., 41 °C), and therefore does not require a thermal cycler. Two isothermal amplification methods including nucleic acid sequence-based amplification (NASBA) and Loop mediated isothermal amplification (LAMP) have been used for the detection of norovirus and HAV in different food matrices (Jean et al. 2004; Abd el-Galil et al. 2005; Fukuda et al. 2008).

3.5.1. Nucleic Acid Sequence-Based Amplification (NASBA)

NASBA technology was developed by J. Compton in 1991 and is also known as "self-sustained sequence replication" (3SR) and/or Transcription mediated amplification (TMA) (Compton 1991; Gill and Ghaemi 2008; Guatelli et al. 1990). The reaction is performed at a single temperature, normally 41 °C. At this temperature, the genomic DNA from the target microorganism remains double-stranded and does not become a substrate for amplification (Sergentet et al. 2008). A NASBA reaction mix consists of avian myeloblastosis virus reverse transcriptase (AMV-RT), T7 RNA polymerase and RNase H with two oligonucleotide primers. This approach makes use of two specific primers flanking the sequence to be amplified. The first primer (P1) carries the sequence specific for the T7 RNA polymerase at its 5′ end and is used to initiate the RNA reverse-transcription (RT) reaction, catalyzed by a reverse-transcriptase. The RNA strand in the RNA–DNA hybrid, resulting from the RT reaction, is then degraded by RNAse H. The remaining cDNA is accessible to the second primer (P2), which initiates the synthesis of the complementary strand. A third enzyme, T7 RNA Polymerase, docks the double strand DNA on the sequence at the 5′ end of P1, thereby producing many RNA copies of the sequence of interest (Fig. 11.7). The cycle of first strand synthesis/RNA hydrolysis/second strand synthesis and RNA transcription, is repeated indeterminately starting from the newly transcribed RNA. RNA and double strand cDNA of appropriate size accumulate exponentially and can be separated and detected by agarose gel electrophoresis.

3.5.2. Molecular Beacon in NASBA

Molecular beacons are DNA oligonucleotides with a hairpin loop sequence labeled with a fluorophore at the 5′ end and a quencher at the 3′ end (Tyagi and Kramer 1996). The sequence of the 3′-end of the molecular beacon is complementary to that of the 5′ fluorophore end and a hairpin stem is formed. The hairpin loop sequence is complementary to the target sequence of the amplicon. In the absence of a complimentary target sequence, the beacon remains closed and there is no appreciable fluorescence. When the loop sequence binds to the target, the hairpin stem opens up and the quencher becomes separated from the fluorophore. The increase in light emitted can be

detected by a fluorometer. Molecular beacons are highly specific to their targets and in a NASBA amplification reaction they form a stable hybrid with their amplified target RNA.

NASBA for HAV, norovirus, astrovirus, and rotavirus assays have been described (Greene et al. 2003; Jean et al. 2001; Jean et al. 2002; Jean et al. 2003; Jean et al. 2004; Moore et al. 2004; Tai et al. 2003) and have been used successfully to detect HAV, rotavirus and noroviruses from contaminated lettuce and blueberry and sliced turkey (Jean et al. 2002; Jean et al. 2003; Jean et al. 2004). Real-time NASBA using molecular beacon probes has been utilized to detect noroviruses in water and ready-to-eat foods (Abd el-Galil et al. 2004; Abd el-Galil et al. 2005; Lamhoujeb et al. 2008; Lamhoujeb et al. 2009).

3.5.3. Loop Mediated Isothermal Amplification (LAMP)

LAMP is a single tube isothermal amplification technique for the amplification of DNA at a constant temperature (between 59 and 65 °C) (Notomi et al. 2000). The amplicons are mixtures of many different sizes of stem loop DNAs with several inverted repeats of the target sequence and cauliflower-like structures with multiple loops (Zhao et al. 2014). LAMP products can be observed with the naked eye by employing SYBR Green I dye (Zhao et al. 2014). A two-step isothermal amplification assay was also developed that combined NASBA and LAMP for rapid and sensitive detection of noroviruses in oysters (Fukuda et al. 2008). Compared to PCR, LAMP is a simple low-cost molecular detection method which has the potential to be used as a simple point of care diagnostic test. Because LAMP is isothermal no expensive thermocyclers are needed and therefore this method could be particular useful for infectious disease diagnosis in low and middle income countries.

4. CONCLUDING REMARKS

For the past 2 decades, significant improvements have been made in methods used for the detection of foodborne viruses in various food matrices. Advances in molecular techniques e.g., RT-PCR, real-time PCR, microarray, biosensors, and next generation sequencing (NGS) have now made the detection of HAV and norovirus more feasible. Real-time PCR detection of viral nucleic acid offers one of the best approaches for the sensitive detection of foodborne viruses in food matrices that are linked epidemiologically with acute gastroenteritis or hepatitis. In recent years NGS methods have opened new avenues for sensitive detection of viruses in food and clinical samples as they have the potential to become the gold standard laboratory method to investigate foodborne outbreaks once their sensitivity is improved for the detection of low copy-numbers of virus.

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CHAPTER 12

Methods for Estimating Virus Infectivity

Doris H. D'Souza

1. INTRODUCTION

Foodborne viruses of human health concern include human noroviruses, hepatitis A virus, rotaviruses, hepatitis E virus, Aichi virus, adenoviruses, parvoviruses, astroviruses, coxsackie viruses and other small round structured enteric viruses. These viruses can be observed using Transmission Electron Microscopy (TEM), but whether the viral particles are infectious or not cannot be determined by TEM. The determination of the presence of infectious virus is crucial in the food industry as well as clinical and environmental studies to control viral spread.

Historically, infectious virus titers were estimated by observation of cytopathic effects (CPE) using confluent host cells grown in cell-culture plates in laboratories. Available host cells and cell-culture systems include fetal rhesus kidney (FRhK-4) cells for lab-adapted hepatitis A virus (HAV), monkey kidney epithelial cells (MA-104) for rotavirus, Vero Cells for Aichivirus, and human embryonic kidney cell line 293A and human lung carcinoma cell line A549 for enteric adenovirus 40 and 41, etc. Primarily, 50 % tissue culture infectious dose ($TCID_{50}$) or plaque assays (for viruses that form plaques) were and continue to be used to estimate infectivity as a gold standard (Hamza et al. 2011). The virus titers are estimated by preparing serial dilutions (usually tenfold) of the sample and infecting the respective confluent host cell lines either in 96-well plates (using 3–4 wells per dilution) for $TCID_{50}$ assays or in 6-well plates for plaque assays. This is followed by incubation at 37 °C under 5 % CO_2 for various time periods ranging from 2 to 7 days and then visualization of CPE under an inverted microscope and of plaques by the naked eye. The number of wells per dilution that show CPE is recorded and the titer calculated using one of two formulas (Karber 1931; Reed and Muench 1938).

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For the viruses that form plaques, the zones of clearing are recorded where each zone /hole/plaque is considered equivalent to a single plaque forming unit (PFU). The number of plaques obtained is multiplied by the dilution factor to obtain the viral titer. A single infectious virus is capable of infecting a single susceptible host-cell to form a plaque; however, an aggregate (viruses can form aggregates in suspension), that may contain two to hundreds of infectious virions, can also infect a single susceptible cell or form a single plaque (Knight et al. 2013).

The particle-to-PFU ratio is described as a measure of the fraction of virus particles in a sample that can complete an infectious cycle, and this ratio has been reported to be as high as 10,000:1 for many animal viruses (Knight et al. 2013). This high particle to-PFU ratio may occur due to: the presence of defective viral particles with minimally damaged capsids (which cannot be determined by EM visualization); genomes that are either incomplete or damaged, aggregation of viral particles (aggregation is pH dependent and affects infectivity) in suspension; or blockage of replication even if the genome is released into the host cell after successful attachment and entry (as has reported for poliovirus) (Brandenburg et al. 2007; Knight et al. 2013). These and other researchers find it complex to define an "infectious" virus as they suggest these factors described above need to be considered when determining/evaluating "infectivity" by a particular detection method.

Also, these cell-culture based infectivity assays have drawbacks of being labor-intensive, time-consuming (can take up to a week), and expensive. Moreover, in the current absence of available reproducible cell-culture systems for human noroviruses (HuNoVs) and the wild-type hepatitis A virus (HAV), infectivity of the viruses is determined using modifications of conventional molecular assays. The lack of cell-culture based infectivity assays is reported to have hindered the development and verification of appropriate control measures for use in hazard analysis and critical control point (HACCP) systems and in pre-requisite programs for HuNoV and for overall foodborne virus control during food processing, transport, handling, sale and delivery (Topping et al. 2009).

For the detection of contamination of food, water or environmental samples with enteric viruses, molecular methods have been developed that can detect low viral load that can cause infection. However, it has been argued/debated that molecular-based approaches can detect the viral nucleic acids of both infectious and non-infectious viruses. Hence, the detection of inactivated viruses by molecular-based assays after employment of processing treatments and inactivation methods may provide misleading/confusing results. Novel methods to determine and estimate viral infectivity after treatments are, therefore, being investigated. This chapter will provide a brief overview of the current research trends and approaches for the determination of infectivity of primarily the currently non-cultivable HuNoVs and HAV as summarized in Table 12.1.

Table 12.1 Representative methods used to determine infectivity of viruses

Method	*Representative examples*	*Representative references*
Traditional cell culture-based infectivity assays	TCID50 assays/ plaque assays	Hamza et al. (2011), Karber (1931), and Reed and Muench (1938)
Molecular detection after measures to assess capsid integrity	Enzymatic pre-treatments	Nuanualsuwan and Cliver (2002), Ronnqvist et al. (2014), Topping et al. (2009), Nowak et al. (2011a, b), Escudero et al. (2014), etc.
	Intercalating dyes	Parshionikar et al. (2010), Escudero-Abarca et al. (2014), Coudray-Meunier et al. (2013), etc.
	Porcine gastric mucin binding	Kingsley et al. (2014), Li et al. (2011), Li et al. (2013), Dancho et al. (2012), etc.
	Antibody binding assays	Abd el-Galil et al. (2005), Ogorzaly et al. (2013)
	Integrated cell-culture PCR assays	De Chastonay and Siegl (1987), Hyeon et al. (2011), Dunams et al. (2012), De Medici et al. (2001), Cantera et al. (2010), Ganguli et al. (2011), etc.
Cultivable surrogates	Feline calicivirus	D'Souza et al. (2006), Su et al. (2010), Nowak et al. (2011a, b), Fraisse et al. (2011), Hirneisen et al. (2011), and Tung et al. (2013)
	Murine norovirus	Cannon et al. (2006), D'Souza and Su (2010), Su et al. (2010), Fraisse et al. (2011), Hirneisen et al. (2011), and Tung et al. (2013)
	Tulane virus	Farkas et al. (2008), Hirneisen and Kniel (2013), Tian et al. (2013), etc.
	Porcine sapovirus	Wang et al. (2012)
Virus-like particles (VLPs)	VLP expression systems	Feng et al. (2011), Lou et al. (2012), Caballero et al. (2004), etc.
Animal models	Macaques, mice, gnotobiotic pigs, gnotobiotic calves	Jung et al. (2012), Rockx et al. (2005), Li et al. (2012c), Taube et al. (2013), and Bok et al. (2011)
Human challenge studies	High pressure treated HuNoVs	Leon et al. (2011)

2. REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) FOR INFECTIOUS VIRUS DETECTION UPON PRETREATMENT OF VIRUSES

Since a majority of the foodborne viruses are RNA viruses, RT-PCR has been the chosen method for their detection in clinical, food and environmental samples. This involves conversion of RNA to cDNA by reverse-transcriptases

(Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RTase) or Avian Myeloblastosis Virus (AMV)-RTases are used), followed by PCR amplification. These rapid molecular methods also have disadvantages that include expensive reagents, equipment, and labor as well as the inability to discriminate between infectious and non-infectious virus, as discussed before (Nuanualsuwan and Cliver 2002). Infectivity assays are needed for determination of the effectiveness of a processing technology, sanitation method, or control measure as only infectious virus can transmit disease and outbreaks. As mentioned above, in the absence of suitable cell-culture based systems for the propagation of HuNoVs and wild-type HAV, robust molecular methods that discriminate between infectious and non-infectious virus are being researched. Even though RNA based methods use the principle that mRNA has a shorter half-life than DNA, they cannot conclusively discriminate between infectious and non-infectious viruses (Parshionikar et al. 2010).

To estimate infectivity using molecular approaches, many researchers have directed studies to determine viral capsid integrity prior to RT-PCR, as both the virus capsid and nucleic acid/genome are required for infectivity. This is based on the fact that infectious virus should have an intact capsid that can attach to host cells, bind to receptors, as well as protect the nucleic acid from damage by nucleases and various other environmental conditions (Nowak et al. 2011a, b; Knight et al. 2013). Thus, damage to the viral capsid can lead to loss of infectivity.

2.1. Enzymatic Pretreatments for Detection of Damaged Capsid/ Loss in Infectivity

It is hypothesized that a virus with an intact and unaltered capsid should contain an intact genome and should not be affected by enzymatic treatments targeting the internal nucleic acids. Therefore, molecular amplification of such viruses should yield positive products even after certain enzymatic treatments (such as with ribonuclease treatment that should only destroy released or free RNA) are applied. Enzymatic pre-treatments (such as with ribonuclease (RNase)) are being researched and used in combination with (prior to) RT-PCR to estimate viral infectivity after various food processing and inactivation treatments (Nowak et al. 2011a, b; Li et al. 2012b; Nuanualsuwan and Cliver 2002; Topping et al. 2009; Escudero-Abarca et al. 2014). If virolysis or capsid damage results after a food processing or inactivation treatment is applied, the nucleic acid genome of the virus will be exposed and hence susceptible to attack by the RNase (or DNAse for DNA viruses) which will destroy the nucleic acid material. Therefore, product amplification is either not obtained or decreased following RT-PCR (or PCR) or RT-qPCR (real-time quantitative RT-PCR as determined by reduced signal). Appropriate positive, negative, and internal amplification controls are used to determine assay robustness.

Escudero-Abarca et al. (2014) used RNase pretreatment followed by RT-PCR on monodispersed Snow Mountain Virus (SMV, a prototype HuNoV

genogroup II) suspensions that were heated at 77, 80, 82, and 85 °C for various time intervals in order to estimate capsid integrity as an alternate virus infectivity assay. After thermal treatment of the viruses, these researchers added commercial RNase and incubated the mix at 37 °C for 15 min, followed by volume adjustment with phosphate buffered saline, subsequent RNA extraction and detection by real-time RT-PCR (TaqMan assay). They reported D-values (time required to cause a one-log reduction in infectivity) of 16.4 ± 0.4, 3.9 ± 0.2, 0.9 ± 0.3, and 0.12 ± 0.00 min at 77, 80, 82, and 85 °C, respectively for RNase-treated SMV. In contrast, the viral RNA levels remained constant irrespective of the time-temperature combination when evaluated by traditional RT-qPCR in the absence of enzymatic treatment. Thus, they showed the suitability of using RNase treatment prior to RT-PCR to estimate infectivity after thermal treatments.

Pretreatment with a combination of proteinase K (20 Units) and RNase (100 ng) for 30 min at 37 °C before RT-PCR was used to determine inactivation of HAV, a vaccine strain of poliovirus 1 (PV-1), and the F9 strain of feline calicivirus (FCV; used as a cultivable surrogate of HuNoV) by three commonly used processing methods: ultraviolet light, hypochlorite and heating at 72 °C (Nuanualsuwan and Cliver 2002). These researchers found that viruses which were inactivated by these treatments were not detectable by agarose gel electrophoresis following RT-PCR. They had added RNase inhibitor solution (40 units) to the viral solution after the enzymatic treatment (to stop the RNase activity to allow for amplification in the RT-PCR assay), followed by RT-PCR of both inactivated and inactivated viruses. For PV-1, they reported that there was no virus detectable by RT-PCR when combined with enzymatic RNase and proteinase K pre-treatments following virus exposures with UV doses of 180 mWs/cm2, free chlorine (hypochlorite) at 2.15 mg/l-min or heating at 72 °C for 17 s. Use of RNase or proteinase K treatments individually following UV and hypochlorite inactivating treatments was shown to result in similar positive HAV RT-PCR results when compared to the non-inactivated enzyme-treated HAV controls, indicating that the combined RNase and proteinase K treatments were needed to estimate inactivation. For HAV, these authors showed that there were no detectable RT-PCR products after inactivation of initial titer of 10^3 PFU virus by treatments with UV at doses of 252 mWs/cm2, free chlorine (hypochlorite) at 21 mg/l-min or heating at 72 °C for 56 s, while the non-inactivated, enzyme treated viruses showed positive RT-PCR results. They also showed that there were no detectable RT-PCR products after inactivation of 10^3 PFU of FCV by treatments with UV at doses of 357 mWs/cm2, free chlorine at 1.2 mg/l-min or heating at 72 °C for 23 s using the combined enzymatic treatment followed by RT-PCR (Nuanualsuwan and Cliver 2002).

Based on their results with UV-treatment of HAV, Nuanualsuwan and Cliver (2002) hypothesized that the coat protein of UV-inactivated viruses could become partially unfolded after UV-exposure, such that the viral RNA is still protected. However, the coat protein eventually becomes susceptible to proteinase K (that degrades the capsid protein and destroys the intact viral

structure, which then allows RNase to digest and degrade the viral RNA inside the particles) (Nuanualsuwan and Cliver 2002). They also showed that HAV maintained the integrity of the capsid to a greater extent when heat inactivated and especially when hypochlorite treated, as both enzymes (proteinase K and RNase) were needed to obtain negative RT-PCR results.

However, no clear correlation under relatively mild inactivation conditions was obtained with this pre-enzymatic treatment technique indicating that, in cases of mild inactivation, RNA might continue to experience some degree of residual protection by the capsid (Nuanualsuwan and Cliver 2003; Baert et al. 2008a). The lack of a clear advantage of RNase protection methods (for the detection of active infectious virus) in these cases over direct RT-PCR may have prevented their adoption and application in studies of virus response to mild treatments (Baert et al. 2008a). Since these methods appear to be advantageous mainly under extreme, severe or harsh inactivation conditions, the use of molecular assays to determine infectivity depends on the process and degree of the inactivation method and hence the results obtained from these molecular assays must be interpreted with caution.

Topping et al. (2009) also suggested that the apparent disconnection between the lack of RNA destruction and loss of infectivity under mild conditions may be due to the combination of the methods of inactivation used and a consequence of residual RNA protected from attack by RNases. In the past, some researchers used samples with high viral copy numbers or high virus titers (~≥5 log units) to determine large reductions in viral RNA (Nuanualsuwan and Cliver 2002). However, under relatively mild conditions, having high initial virus titers may lead to only a very small reduction in nucleic acid amplification as determined by RT-PCR and gel electrophoresis or threshold cycle (Ct) values when real-time molecular detection approaches are used. These values have been suggested to actually correspond to large differences in amplified copy numbers because of the exponential nature of the PCR, which can be detected more sensitively at low viral titers or copy numbers (Topping et al. 2009). For example, when the Ct values are too low and close to each other due to the high copy number, the detection range is not broad. However, when the copy number is low, the Ct values are high and hence within this range of high Ct values smaller differences are easier to detect (it is easier to differentiate between 34 and 31 and 29, than 6.5, 7, 7.5). They used this approach to determine the thermal inactivation of FCV-F9 in terms of loss of capsid integrity (RNA exposure) and also by infectious plaque assay and compared the RT-qPCR results for FCV-F9 with that of thermally treated GII.4 HuNoVs. Briefly, they heated 50–100 µl of FCV-F9 and three unlinked clinical isolates of HuNoV GII.4 diluted samples containing 10^4–10^5 RT-qPCR copies for 2 min at different temperatures (63.3 and 76.6 °C) in thin-walled PCR tubes in a thermal cycler. The RNase ONE enzymatic solution (10 units) was added to the 100 µl (volume adjusted with PBS) thermally treated/inactivated samples and incubated at 37 °C for 15 min (volume adjusted to 140 µl)(Topping et al. 2009). Control samples (heated and unheated) were reported to contain

RNase buffer but without RNase and were stored on ice. They then isolated the RNA using the QIAmp viral RNA kit, followed by detection using TaqMan RT-PCR in a BioRad iCycler. This method was effective in distinguishing between residual RNase-resistant or protected RNA that is present with inactivated viruses and can occur under relatively mild inactivation conditions. This method also enabled them to determine the temperature point at which maximal RNA exposure of infectious virus could be achieved where no amplification was obtained due to RNA being degraded upon exposure to RNase after inactivation (Topping et al. 2009). Their comparative results using FCV-F9 virolysis and infectivity assays after heat treatment showed that a >98.5 % reduction in PCR signal resulting from virolysis was equivalent to a >4 log reduction in infectivity (Topping et al. 2009). Thus similar results of >4 log reduction required for the testing of virucides according to European Standard EN14476.2005 were obtained (Topping et al. 2009; Mormann et al. 2010).

It must also be noted that the exact relationship between infectivity and virolysis is not as straight-forward or clear-cut as described above, since PCR signals can still be obtained in the absence of infectivity (Nowak et al. 2011a, b). Nowak et al. (2011b) also measured capsid destruction or "virolysis" by RT-qPCR in conjunction with RNase ONE treatment (in order to destroy any exposed RNA) of HuNoV GII.4 and FCV-F9 (used as a control) subjected to 5-min treatments with two commercially available alcohol-based hand washes, alcohols (75 % (v/v) ethanol or isopropanol), quaternary ammonium compounds [(0.14 % benzyl ammonium chloride (BAC) or 0.07 % didecyl dimethyl ammonium chloride (DIDAC)], and chlorine dioxide (200 ppm), followed by gel-purification to remove the test virucide. They found that all these treatments at the tested concentrations were ineffective in promoting virolysis of HuNoV GII.4 present in diluted clinical samples, except the combination of heat and alkali treatments (1 M NaOH at 50 °C) (Nowak et al. 2011b).

In another study, virolysis was measured with RNase pretreatment followed by real-time RT-PCR following chlorine treatment. The authors reported >4-log reduction and showed a log-linear relationship between plaque assay results and virolysis when FCV-F9 was treated with 48 and 66 ppm of free chlorine (Nowak et al. 2011a). They also reported that three non-epidemiologically linked HuNoV GII.4 strains from diluted clinical samples showed comparable behavior, but were 10 times more resistant to virolysis than cultured FCV-F9. However, the authors suggested that this was not necessarily due to differences between the viruses, but rather that the clinical matrix of the HuNoV suspension significantly affected the survival of these viruses.

Inactivation of HuNoV GII.4 and murine norovirus (MNV-1, used as a cultivable surrogate for HuNoV) by UV on dry glass surfaces was assessed by RT-qPCR and viability assays with two parallel treatment groups, with one group enzymatically pre-PCR treated with Pronase (a non-specific protease) and RNAse enzymes, and the second group not treated enzymatically (Ronnqvist et al. 2014). A 4-log reduction in MNV-1 titer was observed when the virus was treated with UV doses of 60 mJ/cm^2 or higher. At a dose of ≤150

mJ/cm^2, only a 2-log decline in MNV-1 RNA was observed. They also showed that at higher UV doses of 450–1.8×10^3 mJ/cm^2, the RNA levels of non-enzyme treated samples remained high, at over 1.0×10^3 PCR units (PCRU), while the RNA levels of enzyme-treated samples dropped below 100 PCRU (Ronnqvist et al. 2014). These researchers showed that a significant difference in MNV-1 persistence could be demonstrated when the infectivity assay and enzyme pre-treatment plus RT-qPCR assay were compared. They concluded that genomic detection by molecular methods may overestimate norovirus persistence even when the sample is enzymatically pretreated. Hence, depending on the treatment method, the results obtained by molecular methods should be interpreted accordingly.

Other researchers showed correlation with enzymatic pre-treatment prior to molecular methods and loss of virus infectivity. Pretreatment with RNase followed by reverse transcription-quantitative PCR (RT-qPCR) was used for the detection of Norwalk virus (HuNoV) RNA within intact capsids after the virus was stored in groundwater samples and the authors showed that no significant log reduction of viral RNA was obtained throughout 427 days of storage, though a significant 1.10-log reduction by day 1266 was obtained (Seitz et al. 2011). These researchers also showed that HuNoV spiked in groundwater remained infectious after storage for 61 days at room temperature in the dark as determined by using human challenge studies. Additionally, purified HuNoV RNA was found to persist for 14 days in groundwater, tap water, and reagent-grade water.

Liu et al. (2010) used real-time qPCR with and without prior RNase treatment to determine the efficacy of sodium hypochlorite, ethanol, liquid soap and alcohol-based hand sanitizers against human NoV. In suspension tests, it was found that sodium hypochlorite at ≥160 ppm effectively eliminated the RT-qPCR signal of Norwalk Virus (NV), while treatment with ethanol resulted in only a 0.5 log reduction of NV by RT-qPCR. They found the greatest reduction in RT-qPCR units of NV after finger pads were rubbed with the antibacterial liquid soap (resulting in a 0.67–1.20 log reduction) and when rinsed with water only (resulting in a 0.58–1.58 log reduction), while reductions of only 0.14–0.34 log RT-PCR units were obtained when the alcohol-based hand sanitizer was used on finger pads (Liu et al. 2010). Thus, this study showed the need for improved hand sanitizers to control HuNoV spread (Liu et al. 2010).

Mormann et al. (2010) used RNAse A pre-treatment followed by detection using TaqMan RT-PCR to recover and distinguish between infectious and non-infectious HuNoV GII.3 after various processing/preservation treatments (heating, cooling, cryopreservation) were performed on virus-inoculated solid foods including (fruits, salad, frozen pizza, and frozen pizza baguette), iceberg lettuce, apples, noodle salad, potato salad, tomatoes ketchup, and minced meat. Samples with extracted virus were treated with 35 µg of RNase A (Qiagen) per sample at 37 °C for 1 h followed by the addition of 140 U/sample of Qiagen RNase inhibitor for 30 min at room temperature to stop the RNAse activity. The RNase pre-treatment step was used to avoid false-positive PCR results

caused by non-degraded RNA from disrupted virus particles and thus allowed the detection of intact virus particles (Mormann et al. 2010). These researchers suggested that the established procedure could be applicable for the detection of other non-cultivable enteric and food-borne RNA viruses.

Li et al. (2012b) also evaluated RNase One treatment followed by RT-PCR to determine capsid integrity following heat treatment and found no significant differences between the resulting murine norovirus (MNV-1) viral titers and HuNoV GII.4 titers.

Pre-RNase treatments were also used to eliminate free nucleic acid/genomes and assess viral inactivation after chlorine treatment by subsequent RT-PCR in a study by de Abreu Corrêa (2012). Murine norovirus (MNV-1) showed a 2.5 and 3.5 log genome copy reduction in natural and artificial seawater, respectively, and infectious virus particles were not detected after 30 min of 2.5 mg/liter chlorine treatment (de Abreu Corrêa et al. 2012). These researchers also showed that for human adenovirus 2, reductions of 2.6- and 2.7-log genome copies and 2.3- and 2.4-log PFU were obtained after incubation for 30 min in natural and artificial seawater, respectively. Infectious viral particles were still observed at the end of the assay, and showed a direct correlation between viral genome copies detected after treatment and infectious viral units.

Pre-treatment with RNase exogenously added before detection by RT-PCR of HuNoV GII.4 after 1–14 freeze/thaw (F/T) cycles (–80 °C/+22 °C) or after –80 °C storage for up to 120 days was used to evaluate capsid and viral RNA integrity of HuNoV GII.4 (Richards et al. 2012). These conditions were reported to not cause any alternation to the amount of genomic HuNoV RNA detected, indicating that the viral capsids remained intact as assessed by this method.

Pretreatments with RNase followed by real-time RT-PCR were used to estimate inactivation of HuNoV genogroup I.1 (GI.1) or genogroup II.4 (GII.4) in spiked oyster homogenate samples by high hydrostatic pressure (HHP) at 300–600 MPa at 25, 6, and 1 °C for 5 min (Ye et al. 2014). The authors determined that treatments at 450 MPa at 1 °C could achieve a >4 log reduction of HuNoV GI.1 in both oyster and clam homogenates using this method.

Cell-culture infectivity assays and side-by-side experiments including an RNase pre-treatment (where unaltered or undamaged viral particles are protected and so also termed RNase I protection assay) were used to determine that carvacrol and oregano oil could reduce MNV-1 titers (used as a cultivable HuNoV surrogate) within 15 min by approximately 1 log (Gilling et al. 2014a). They found that carvacrol had greater effects causing a 3.87 log reduction of MNV-1 after 1 h, using the RNase I protection assay suggesting that these compounds/agents acted directly on the virus capsid and subsequently exposing the RNA to degradation from the exposed environment (Gilling et al. 2014a). Thus, they used this method to help understand the mechanism of action of the essential oils being studied.

Gilling et al. (2014b) further tested the efficacies of lemongrass oil, citral, and all-spice oil against MNV-1 using both infectivity assays and RNase I protection assays that resulted in 0.90 log–1.88 log reductions after 6 h, with 3.41 log RT-PCR reductions for all-spice after 24 h, respectively. Based on the RNase I assay, allspice oil appeared to decrease MNV-1 infectivity by acting directly on the viral capsid and RNA (Gilling et al. 2014b).

Modifications of Nucleic Acid Sequence Based Amplification (NASBA)

In addition to RT-PCR, novel isothermal amplification methods such as NASBA have been used to sensitively detect human enteric viruses. The advantage of this method is that expensive thermocyclers are not required, and the reaction can occur at one temperature in a simple water bath. Lamhoujeb et al. (2008) reported the development of a rapid, simple, and sensitive real-time nucleic acid sequence-based amplification (NASBA) assay combined with an enzymatic pre-treatment for distinguishing infectious from noninfectious HuNoV. The assay was subsequently validated using spiked ready-to-eat food samples. These researchers used 22 U of proteinase K freshly dissolved in phosphate-buffered saline for 1 h at 37 °C on inactivated or control HuNoV genogroup II and FCV. They stopped the reaction by adding 2 μl of 200 μM phenylmethane sulfonyl fluoride at room temperature for 30 min. This was followed by the addition of RNase (100 ng) for 1 h at 37 °C and the reaction was stopped by the addition of 80 U of RNase inhibitor solution, prior to RNA extraction, followed by the NASBA assay. When FCV was used as a HuNoV surrogate in the preliminary assays, they reported it to be more sensitive to heat inactivation and enzymatic pre-treatment than HuNoV. These researchers, using this approach, also showed that HuNoV survived on lettuce and turkey for at least 10 days under refrigeration (Lamhoujeb et al. 2008). Thus, the application of enzyme pre-treatment was found to have benefits in estimating infectivity of the non-culturable viruses when using molecular based technologies.

2.2. Labeling with Biotin Hydrazide for Detection of Oxidatively Damaged Viral Capsids

It has been proposed by several researchers that damage to the viral capsid protein can lead to loss of recognition of host-cellular receptors by the viruses and subsequent loss of replication and loss in infectivity. Sano et al. (2010) used the principle of detection of cumulative oxidative damage (by labeling them with biotin hydrazide) on viral particles as an indicator of loss of viral capsid integrity. Amino acids such as lysine, arginine, threonine and proline that are oxidized are known to form covalent bonds with carbonyl groups (associated with loss of protein function). These functional groups can then be labeled with biotin, and the biotin-modified (or damaged) virions can be separated from infectious intact virus particles by avidin-immobilized affinity chromatography

(Sano et al. 2010) and subsequently detected. These researchers treated human astroviruses and HuNoV GII.4 (in phosphate buffered saline) with 1 % sodium hypochlorite (free chlorine) at 4 °C in the dark for 5 and 15 min, followed by neutralization with 1 % sodium thiosulfate and storage at –20 °C. One-ml of these treated viruses were mixed with 25 μL of 50 mM EZ-Link Biotin Hydrazide (Pierce) in DMSO and incubated at room temperature for 2 h with moderate mixing before storage at –20 °C. The thawed virus suspensions were filtered and then passed through an avidin column followed by elution of bound viral particles and testing them for viral infectivity or detection by RT-PCR. These researchers reported that a decrease in astrovirus infectivity positively correlated with oxidative damage. A 2.53 log reduction in infectious titer was obtained with 0.5 ppm chlorine after 15 min, with a corresponding 0.45 log reduction in genome copy numbers. In addition 49.93 % of HuNoV particles were reported to be oxidatively damaged by 1 ppm free chlorine after 15 min (Sano et al. 2010). The authors concluded that this method could be a powerful tool for evaluating the loss in infectivity of non-culturable viruses after oxidative treatments (Sano et al. 2010).

Furthermore, other researchers then applied this technology to detect infectious rotavirus (without the use of cell-culture), where the cumulative carbonyl groups on the capsid protein were biotin-hydrazide labeled, and separated using an avidin-immobilized gel filled spin column (Tojo et al. 2013). Infectious rotavirus titers (as estimated by this method) after treatment with 0.3 mg/L and 0.6 mg/L free chlorine for 3 min were found to be decreased by 0.19 log and 2.6 log, respectively, while no significant reductions in the amplicon copies were reported by these researchers if RT-PCR was applied without the pretreatment method. Using the avidin spin column method for viruses treated by chlorine, a 0.19 log reduction in virus titer was observed. Thus these researchers concluded that this method could be beneficial in estimating viral inactivation by various technologies and methods such as ozone, and oxidizing agents (Tojo et al. 2013).

2.3. Pretreatment with Intercalating Dyes Followed by Molecular Assays for Infectivity Determination

Another approach for determining infectivity by molecular assays is to pretreat the test sample with nucleic acid intercalating agents such as propidium monoazide (PMA) or ethidium monoazide (Graiver et al. 2010; Parshionikar et al. 2010; Kim et al. 2011; Sanchez et al. 2012; Kim and Ko 2012; Coudray-Meunier et al. 2013). Ethidium monoazide (EMA) and propidium monoazide (PMA) are DNA-intercalating dyes with a photo-inducible azide group that covalently cross-links to DNA through visible-light photoactivation (Coudray-Meunier et al. 2013). Both these dyes cannot penetrate intact capsids, but should be able to penetrate damaged or destroyed capsids. Once penetrated, the photo-inducible azide group on PMA molecules covalently cross-links the RNA, producing stable monoadducts that cannot be amplified in subsequent amplification methods such as RT-PCR. Hence, this approach can be used to

determine if the viral capsid has been damaged by various treatments or processing conditions. When this method is coupled to RT-PCR or isothermal molecular amplification approaches, it can enable the discrimination between viable and non-viable microorganisms, albeit only under certain defined conditions of use (Parshionikar et al. 2010; D'Souza et al. 2013; Coudray-Meunier et al. 2013). EMA (ethidium monoazide) and PMA (propidium monoazide) DNA intercalating dyes have been used for treatment of bacterial samples to differentiate between dead and viable cells (Fittipaldi et al. 2012). It has also been reported that PMA is more advantageous and more selective than EMA for dead/non viable organisms as it is relatively membrane-impermeable (Coudray-Meunier et al. 2013).

Pretreatment of inactivated RNA viruses (coxsackie virus, poliovirus, and echovirus) with PMA prior to RT-PCR (PMA-RT-PCR) was used to distinguish between infectious and noninfectious viruses (Parshionikar et al. 2010). However, these researchers reported that PMA-RT-PCR could still detect enteroviruses that were noninfectious after treatment at 19 °C as well as noninfectious Norwalk virus.

Kim et al. (2011) developed an EMA pre-treatment method before RT-PCR for the discrimination of damaged viruses or naked viral RNA from intact viruses that consisted of a dose of 10 μg/mL-EMA with 300 s of light irradiation followed by additional purification by spin-column gel filtration (for the reduction of RT-PCR inhibition by any residual EMA; this can also be carried out using a QIAamp® Viral RNA Mini Kit). They reported that heat-inactivated poliovirus (PV1) showed similar results by EMA-RT-PCR and infectious plaque assay and that the EMA-RT-PCR assay could also be applied for the selective detection of epidemiologically significant water-borne enteric viruses such as enteroviruses and noroviruses (Kim et al. 2011).

Sanchez et al. (2012) used propidium monoazide (PMA) and RNase pretreatments to detect and quantify infectious HAV. They found that 50 μM of PMA and RT-qPCR could be used to selectively quantify infectious HAV in media suspensions. They reported that pre-treatment with PMA was more effective than RNase pre-treatment in estimating the infectivity of thermally inactivated HAV, where the HAV titers were reduced by more than 2.4 log RT-PCR units. They concluded that PMA treatment prior to detection by RT-qPCR is a promising approach to estimate HAV infectivity.

Coudray-Meunier et al. (2013) applied PMA-RT-qPCR to estimate thermal inactivation of HAV and rotavirus (RV) at 37, 68, 72, and 80 °C. They reported maximum decreases for HAV (–1.06 to –1.14 log) and RV (SA11) (–1.60 to –1.71 log) with PMA concentrations ranging from 50 to 100 μM, while for RV (Wa), 75 μM and 100 μM PMA were needed to achieve –1.44 and –1.45 log decreases. On the other hand, using EMA RT-qPCR, maximum decreases were reported for HAV (–1.75 log) with 20 μM EMA, for RV (SA11) (–1.13 log) with 20 μM EMA, and for RV (Wa) (–1.81 log) with 50 μM EMA. These scientists therefore chose optimal concentrations of 20 μM EMA for all the tested viruses (mentioned above), 50 μM PMA for HAV and RV (SA11), and

75 μM PMA for RV (Wa). In addition, they also researched the use of non-ionic surfactants Triton X-100, Tween 20 and IGEPAL CA-630, at 0.1, 0.5 and 1 % for their efficacy in improving the permeability effects of PMA/EMA treatment after incubation for 30 min, 2 h and overnight in the dark followed by exposure to light for 15 min. It was found that pre-treatments for 2 h with 20 μM EMA and IGEPAL CA-630 (0.5 %) were optimal for HAV, 20 μM EMA was optimal for RV (Wa), and 50 μM PMA was optimal for RV (SA11). The study suggested that different capsid structures might be responsible for this difference in monoazide penetration, where some are more susceptible and others more resistant to environmental factors. The study proposed that monoazides bind to stable secondary structures in viral RNA of non-infectious particles with damaged capsids, and thus prevent genome amplification and subsequent detection by RT-PCR (Coudray-Meunier et al. 2013). However, overall these researchers concluded that the effectiveness of combined pre-treatment RT-qPCR assays depended on the target virus and the method of RT-qPCR assay, and that this PMA/EMA assay/approach can be developed for infectious virus determination in food and environmental samples only under specific conditions of inactivation (Coudray-Meunier et al. 2013).

PMA-qPCR was also evaluated for its ability to distinguish between viable and nonviable bacteriophage MS2 after heating at 80 °C for 20 min and also after heating MNV-1 at 72 °C for 10 min (Kim and Ko 2012). The two heat-treated viruses were analyzed by both infectious plaque assays and PMA-RT-qPCR assays. These researchers tested MNV-1 with 125 or 250 μM PMA, and MS2 with 10, 50 or 125 μM PMA for 5 min in the dark with shaking. This was followed by 10 min of light exposure using a 500 W halogen lamp at a distance of 30 cm. Using this procedure, fully-heat-inactivated MS2 was undetectable using either plaque assay or the combined PMA-qPCR assay, while greater than 80 % of inactivated MS2 could still be detected when conventional real-time PCR without the PMA pre-treatment was used (Kim and Ko 2012). Based on their results, they suggested that combined PMA-RT-qPCR was suitable for the differentiation of heat-inactivated and non-inactivated MS2. However these researchers also reported that heat-inactivated MNV-1 could still be detected after treatment with either 125 or 250 μM PMA by the PMA-qPCR assay (Kim and Ko 2012). They attributed these results to differences between virus inactivation procedures and the mechanism of inactivation associated with differences in virus structure where heating at high temperatures (at 80 °C for MS2) is known to damage the viral capsid (Parshionikar et al. 2010). Thus overall, Kim and Ko (2012) concluded that that the combined PMA-RT-PCR assay had limited applicability as it could only partially distinguish between viable and heat-inactivated MNV-1. They suggested that improvements (characterization of effects of particulate matter in clinical and environmental samples) and optimization of the technique are needed for the detection and quantification of infectious MNV-1 and other viruses.

Heat-treated, monodispersed Snow Mountain Virus (SMV) was reported to produce D-values of $25.6 \pm 2.8, 3.1 \pm 0.1, 0.7 \pm 0.04$ and 0.2 ± 0.07 min at 77, 80,

82 and 85 °C, respectively when PMA pre-treatment was used prior to RT-qPCR (Escudero-Abarca et al. 2014). However, lower D-values of 16.4 ± 0.4, 3.9 ± 0.2, 0.9 ± 0.3 and 0.12 ± 0.00 min were obtained after thermal treatments at 77, 80, 82 and 85 °C, respectively, as assessed by RNase pre-treatment prior to RT-qPCR (Escudero-Abarca et al. 2014). These authors concluded that nucleic acid intercalating agents together with RT-qPCR can be used to estimate HuNoV capsid integrity as an alternate for estimating infectivity after thermal inactivation treatments. From this study, they also concluded that the cultivable surrogate viruses appear to be more sensitive to heat than the HuNoVs (Escudero-Abarca et al. 2014).

An EMA-based PCR was also reported to be unable to distinguish between infectious and non-infectious avian influenza virus particles (Graiver et al. 2010). Therefore, these authors advised that the results obtained from these molecular based detection assays need to be interpreted with caution.

2.4. Porcine Gastric Mucin (PGM) as a Method for Selective Binding of Intact Viral Capsids

Human histo-blood group antigens (HBGAs) present on intestinal epithelial cells and in saliva are considered to be attachment factors and possible receptors for human noroviruses (HuNoVs), reportedly necessary for host infection (Tan and Jiang 2005). Studies have shown that individuals lacking the fucosyl transferase (*fut2*) gene for the expression of H type antigens, also known as non-secretors, may be resistant to HuNoV infections (Marionneau et al. 2002). Additionally, it has been reported in literature that HuNoV and its surrogates have the ability to bind to HBGAs in porcine gastric mucin (PGM) and sialic acid (Tian et al. 2008; Hirneisen and Kniel 2012). PGM contains A-type, H type-1, and Lewis b HBGAs (Tian et al. 2008). Tian et al. (2005) in their study first reported the ability of recombinant norovirus virus-like particles (rNVLP) to bind to PGM and also showed that PGM binding competitively inhibited their binding to HBGAs. This finding generated a great interest in the research community that has also triggered research into the possibility of utilizing gastric mucins as antivirals (Lieleg et al. 2012). The binding ability of HuNoV to PGM has been widely exploited and used for the recovery of virus from complex matrices such as stool as well as food samples, for the evaluation and determination of the mode of action of antiviral treatments, and in assays to discriminate between infectious and non-infectious virions (Dancho et al. 2012; Knight et al. 2013).

The PGM assay is based on the similar principle of detection of intact viral capsid as used for intercalating dyes and enzymatic treatments. As reported earlier, the genome of HuNoV is protected by its capsid, and any damage to the capsid makes the viral RNA prone to degradation by RNases (purposefully added in the laboratory or innate in the environment) or susceptible to intercalation by dyes (Knight et al. 2013). PGM binding assays are based on the principle that only the viruses with intact capsids will bind to the PGM and thus only viruses with intact RNA will be detected after PGM binding is fol-

lowed by RT-PCR (Li et al. 2011). This binding is said to mimic the host receptor-binding step in viral infection (Richards et al. 2012), since successful infection of host cells requires an intact receptor-binding site on the viral capsid and an intact capsid is required to maintain the integrity of the viral RNA.

Since PGM contains HBGAs, including some which can bind HuNoVs, decreased binding or lack of binding to PGM can be considered an indication of loss of HuNoV infectivity (Ye et al. 2014). Use of the PGM binding step followed by molecular detection has been suggested to be a useful tool to differentiate between infectious and non-infectious viral particles after inactivating treatments, but its efficacy in doing this may depend on the processing conditions used. Common processing methods such as heat, UV, and high pressure are hypothesized to inactivate viral particles through damage to their capsid, rendering them non-infectious. Hence the PGM assay may be useful for estimating virus infectivity using these treatments (Li et al. 2011; Li et al. 2013; Kingsley et al. 2014).

The effect of parameters such as pH and incubation time on the binding efficiency of HuNoV (GI and GII) to PGM coated magnetic beads were evaluated by researchers (Tian et al. 2010). These researchers found that the PGM binding to viruses is rapid that occurs within 15 min of incubation, and that this binding of HuNoV can be enhanced at lower pH. PGM comprises of essentially glycoproteins with an isoelectric point near 2.0 and thus are negatively charged around pH 3.0–4.0 (Cao et al. 1999). As the isoelectric point of HuNoV GI and GII falls within the range of 5.0–6.0, the viral capsid proteins will have a positive charge at the low pH of 3.0 (Goodridge et al. 2004). The authors therefore suggested that the opposite charge attraction at lower pH enhances the binding efficiency of HuNoV to PGM that can be used to extract intact viral particles for their detection by subsequent molecular assays or cell-culture assays as appropriate (Tian et al. 2010).

PGM binding followed RT-PCR detection was also used to distinguish between infectious and non-infectious HuNoVs (G.I and G.II) and MNV-1 after heat and hydrogen peroxide treatment (Li et al. 2011). These authors coated 96-well plates with receptors (ganglioside GD1a) for MNV-1 and PGM (2 ug/well) for HuNoVs in carbonate buffer overnight at 4 °C, followed by blocking with 1 % BSA for 1 h at 37 °C, and then incubation with virus samples for 1 h at 37 °C. The plates were washed and RNA was extracted by direct addition of lysis buffer from the RNeasy Mini extraction kit and detected by RT-PCR. For HuNoV treatment with 2.1 % hydrogen peroxide (H_2O_2) for 5 min, decreases of >2.5 log RT-PCR units for GI.8 and 1 log RT-PCR units for GII.4 were reported when RT-PCR was used alone, while using the PGM-binding assay prior to RT-PCR showed reductions of >1.7 log for GI.8 and >1.6 log for GII.4 (Li et al. 2011). They also reported that HuNoVs were more resistant to heat at 70 °C for 3 min than 2.1 % H_2O_2 for 5 min, where only up to a 1 log reduction of GI.8 HuNoV was obtained after heat treatment and RT-PCR alone, and with no significant reduction in detected virus using the PGM-binding plus RT-PCR assay. There was no significant reduction in

genome copies (RT-PCR units) detected by these researchers for HuNoV GII.4 by RT-PCR alone; although ≥1 log reduction was reported using the PGM-binding RT-PCR assay after heat treatments. When MNV-1 was treated with 2.1 % H_2O_2 for 5 min or heated to 70 °C for 3 min, 6 log viral reduction was obtained as determined by the infectious plaque assay while analysis with the receptor-binding plus RT-PCR assay showed a reduction of only up to 3 log. They suggested that this difference could be attributed to low affinity non-specific binding of the viral capsid to the receptor molecules on the plate (Li et al. 2011). Hence, the limitations of this method should also be noted.

Other researchers determined the effect of thermal, ultraviolet light and high hydrostatic pressure treatments on binding abilities of HuNoV GI and GII to PGM and found that heat-inactivated HuNoV had reduced binding ability to PGM compared to untreated virus (Dancho et al. 2012). This study showed that HuNoV GI.1, after heating for 1 min at 60 °C, decreased its PGM binding to 6 % and was further reduced to negligible PGM binding after 2 min (Dancho et al. 2012). These results translated to an average 0.9 log and 3.1 log reductions of bound HuNoV to PGM-magnetic beads (PGM-MBs) after thermal treatment for 60 and 120 s, respectively, when compared to untreated HuNoV binding to PGM. Furthermore, these researchers showed that UV treatments of 1 J/cm^2 caused decreases in HuNoV PGM-binding to 4 %, with a reduction of PGM-binding to undetectable levels after treatment with 2 J/cm^2. These researchers also showed that HPP treatments of 600 MPa at 5 °C could reduce GI.I and GII binding to 0.3 and 4 %, respectively from initial binding levels of 71 and 69 %. Thus, they suggested that the PGM binding assay could determine the extent of capsid damage caused by all three commonly used food processing methods that could render the virus non-infectious and prevent any further transmission.

The PGM binding assay was also applied to determine the effect of freeze-thaw cycles ((−80 °C/+22 °C) or after −80 °C storage for up to 120 days) on the capsid integrity and viral RNA persistence of HuNoV GII.4, where no significant change in the integrity of the virus capsid or infectivity was reported after 14 freeze thaw cycles (Richards et al. 2012). This study was undertaken because clinical samples obtained from outbreaks are often frozen until use and may undergo repeated freeze-thaw cycles that could potentially cause viral capsid damage, as suggested by these authors. They also hypothesized that slow thawing at room temperature as opposed to rapid thawing could cause a greater damage to the capsid due to ice crystal formation (Richards et al. 2012).

MNV-1 attachment to host cell receptors and its PGM-binding properties after heat, high hydrostatic pressure, ozone and UV treatments were correlated with infectivity using the MNV-1 plaque assay (Hirneisen and Kniel 2012). These researchers coated ELISA plates with PGM, followed by the addition of either untreated, heat-, high pressure-, ozone-, or UV-treated MNV-1. Bound virus was detected after the addition of monoclonal anti-MNV IgG primary antibody, binding of the horseradish peroxidase (HRP)-

conjugated goat anti-mouse IgG secondary antibody, using 3,3,5,5-tetramethylbenzidine (TMB) liquid as the HRP substrate at RT for 5 min, and measuring absorbance at 405 nM. They found that heat-treated MNV-1 attachment to PGM decreased significantly and corresponded directly with decreasing viral infectivity after thermal treatments at 80 and 100 °C for 5 min. These findings also correlated with the results obtained by RNase pre-treatment followed by molecular detection (Hirneisen and Kniel 2012). However, no significant difference in attachment to PGM was found among pressure-, ozone- or UV-treated MNV-1. Such findings could be attributed to different modes of action on the viral capsid by the various food processing technologies. Therefore, it was concluded that this PGM-ELISA method can only estimate the infectivity of MNV-1 after heat-treatment. Also, as UV is known to mainly target nucleic acids and may not necessarily affect the binding properties of the capsid, it is not surprising that the binding and attachment properties of MNV-1 were not affected by UV treatment (Nuanualsuwan and Cliver 2003).

The PGM-binding assay also helped determine the effects of high hydrostatic pressure (HHP) treatment on viral infectivity and capsid damage for HuNoV (Li et al. 2013). High hydrostatic pressure (HHP)-treated HuNoV strains, GI.1 did not show any reduction in PGM-binding after exposure to 450 MPa pressure at 21 and 35 °C, but with the same pressure at lower temperatures (1 and 4 °C) reductions of 3.0 and 1.8 log (RT-PCR units) were observed. A similar trend was observed for HuNoV GII.4 strains, where treatments with 250 MPa did not result in any significant PGM-binding reduction at 21 and 35 °C, while at lower temperatures of 1 and 4 °C, reductions in RT-PCR units reached approximately 2 log following PGM-binding (Li et al. 2013). Furthermore, when these researchers spiked viruses on dry blueberries and treated the berries with 600 MPa pressure at 1 and 21 °C, reductions of less than 1 log were observed, whereas a 2.7 log reduction was reported for viruses spiked on wet blueberries (Li et al. 2013). The authors suggest that this difference between the wet and dry states could be due to greater water pressure, causing viral capsid damage by disruption of hydrophobic interactions. High hydrostatic pressure treatments are reported to cause capsid damage and thus the PGM-binding assay can be a useful tool in determining the extent of capsid damage and loss of infectivity (Kingsley et al. 2014).

The PGM-binding method was also used to distinguish between infectious and non-infectious GI.1 and GII.4 HuNoVs from inoculated oyster homogenates that were high hydrostatic pressure (HHP) treated at 300–600 MPa at 25, 6, and 1 °C for 5 min (Ye et al. 2014). These researchers first applied an RNase pre-treatment to the HHP-treated oyster homogenates and viral particles were extracted with PGM-conjugated magnetic beads (PGM-MBs), followed by quantification of viral RNA by real-time RT-PCR. HuNoV RNA obtained from oysters was reported to be reduced by 0.4 to >4 log RT-PCR units after HHP treatments at 300–600 MPa using the PGM-MB binding method prior to RT-PCR assay, with HuNoV GI.1 being more resistant to HHP treatment than

HuNoV GII.4 (Ye et al. 2014). The authors concluded that this method could be useful in distinguishing between infectious from non-infectious HuNoV subjected to HHP, and that HHP was a useful method to inactivate HuNoV in shellfish.

More recently, the PGM-binding assay was used to evaluate the inactivation of HuNoV in stool samples after chemical treatments (Kingsley et al. 2014). Chlorine treatments of 33 and 189 ppm were shown to cause 1.48 and 4.14 log reductions HuNoV binding to PGM, while 5 % trisodium phosphate treatments were reported to reduce HuNoV binding by 1.6 log. However, these researchers reported that aqueous chlorine dioxide did not have any significant effect on HuNoV binding to PGM. This study suggested that chemical sanitizers like chlorine could possibly cause damage to the viral capsid, thereby causing a loss in the binding capabilities of the viral particles. The researchers, however, advise that the use of the PGM-binding assay prior to RT-PCR may not be sufficient in examining the exact nature or degree of capsid damage occurring after chemical treatment, as some types of capsid damage may not prevent PGM binding but could alter regions of the viral capsid not involved in PGM binding. They suggest using other available techniques such as nucleic acid intercalating dyes (PMA or EMA as discussed above) and/or other visual methods such as electron microscopy to get a better overview of the nature and degree of capsid damage.

Thus far, PGM has been extensively studied for its ability to bind HuNoVs. PGM can be a suitable alternative for examining viral capsid damage and assessing the infectivity of HuNoVs after virucidal treatments or after application of certain processing and control strategies until a reproducible, robust *in vitro* HuNoV cell culture infectivity assay is developed.

2.5. Other Binding-Based Infectivity Assays

Immunomagnetic separation targets surface epitopes of viral capsids using antibodies bound to magnetic beads. Using this method, viruses with an intact antibody-recognition site are recovered which could correlate with those viruses that have an intact capsid and are therefore potentially infectious. Immunomagnetic separation (IMS) along with molecular beacon (MB) real-time RT-PCR was applied for the detection of infectious hepatitis A virus (HAV) from ground water samples with a detectable signal for 20 PFU of HAV (Abd el-Galil et al. 2005).

A similar immunocapture real-time PCR assay (IC-qPCR) method was used to detect the presence of infectious, intact human adenovirus (HAdV) types 2 and 41 in river water samples (Ogorzaly et al. 2013). Using this IC-qPCR approach, a 90 % decrease in positive signal detection was observed after HAdV-2 was heat-treated at 95 °C for 5 min. These researchers used DNAase treatment to avoid detection of viral particles without capsid protection, and found that IC-qPCR could distinguish between intact and damaged particles with a sensitivity of 10 most probable number cytopathic units (MPNCU)/reaction for the IC-qPCR assay (Ogorzaly et al. 2013).

2.6. Cell-Culture Combinations with Molecular Based Detection (RT-PCR)

Cell culture assays combined with molecular assays have been proposed for the rapid detection of infectious human enteric viruses. This procedure involves the initial propagation of infectious virus particles in cell culture (ICC) followed by the detection of negative strand RNA replicative intermediates using strand-specific RT-PCR (Jiang et al. 2004). The principle behind using this method for detection is that during the replication of positive-sense ssRNA viruses, a negative-strand RNA intermediate is formed that serves as a template for the synthesis of positive strands. During active replication in host cells, both negative- and positive-sense viral RNA strands can be detected (Siegl et al. 1984; De Chastonay and Siegl 1987). HAV from lettuce was extracted and concentrated using a positively charged membrane (NanoCeram matrix) followed by detection using ICC-RT PCR. This method was reported to be faster, where positive samples were detected within 2 days as compared to the 7 days needed for detection by traditional cytopathic assays (Hyeon et al. 2011).

An integrated molecular and cell-culture based approach was also applied to detect the presence of infectious HAV in naturally contaminated mollusks, where these mollusks were tested for HAV RNA using RT-nested PCR and confirmed using host infection of positive samples by cell-culture based assays (De Medici et al. 2001). The study reported the presence of HAV RNA in 34 % of the tested samples; however, only 12.7 % of the total samples were reported to be positive for the presence of infectious virus by cell culture (De Medici et al. 2001).

The simultaneous and rapid detection of multiple infectious viruses in one cell line (A549 cells) using molecular beacons was also developed (Dunams et al. 2012). These researchers constructed the molecular beacon backbone with sulfur (substituting for nonbridging oxygen) and a 2=–sugar deoxy with a 2=–O-methyl group for resistance to attack/degradation by nucleases. They reported that the probes used were target-specific without any non-specific binding to non-target genomes. Using this method, they reported detection of 1 PFU each of echovirus 17 and adenovirus 2 within 5 and 7 h, respectively, as compared to the respective 48 and 168 h normally required by the traditional/conventional plaque assays for these viruses. They also reported there was a direct correlation between the plaque forming units obtained by the cell-culture based assay and the number of virus-infected fluorescent cells (as visualized by fluorescent microscopy) after molecular beacons were included. Therefore, the combined cell culture-based molecular beacon assay was suggested as a potentially useful method for the rapid detection of infectious enteric viruses (Dunams et al. 2012).

Cantera et al. (2010) used engineered buffalo green monkey kidney (BGMK) cells expressing the cyan fluorescent protein (CFP)-yellow fluorescent protein (YFP) substrate that undergoes fluorescence resonance energy transfer (FRET) upon cleavage by the viral 2A protease (2A(pro)) to rapidly

detect infection by poliovirus 1 (PV1) (Cantera et al. 2010). These researchers found that infected cells with an increased CFP-to-YFP ratio could be detected at 5 h post-infection, though detection of 1 PFU required 12 h infection. Similar results were obtained with this new approach when compared to traditional plaque assays performed on spiked environmental samples.

A single infectious MNV-1 viral particle was reported to be detected within 6 h in RAW 264.7 cells by use of a TAT peptide-delivered molecular beacon, which was a 12-fold improvement in the time required for detection and quantification of MNV-1 compared to the conventional plaque assay (Ganguli et al. 2011). Thus, direct detection of infectious virions in cell-culture was observed without the need for amplification.

3. USE OF CULTIVABLE SURROGATES FOR THE DETERMINATION OF HUNOV INFECTIVITY

3.1. Feline Calicivirus as a Cultivable HuNoV Surrogate to Determine Infectivity

Feline calicivirus (FCV) of the genus *Vesivirus* within the *Caliciviridae* family is a major cause of upper respiratory tract infection in cats, typically characterized by oral ulcerations, limping syndrome, and ocular and nasal discharge (Thiel and Konig 1999; Doultree et al. 1999). It is similar to HuNoV in being a small, round, non-enveloped, single-stranded RNA virus with a 7.7 kb genome, containing 3 ORFs. Since it was the first calicivirus reported to be cultivable in the laboratory in Crandell Reese feline kidney (CRFK) cells and due to its relatedness to HuNoV, it was widely used as a surrogate for HuNoVs to determine the effectiveness of various treatment processes and its persistence in the environment (D'Souza et al. 2006; Su et al. 2010; Nowak et al. 2011a, b; Fraisse et al. 2011; Hirneisen et al. 2011; Tung et al. 2013). It is transmitted through air, via fomites, and via nasal, oral or conjunctival routes (Radford et al. 2007). However, since it is transmitted via a respiratory route, it does not mimic HuNoV behavior during transmission and it is believed to be less environmentally stable than HuNoV. In addition, since unlike HuNoV, FCV is sensitive to extremes in pH (low pH of 2.0–4.0 and high pH 10.0) (Cannon et al. 2006), it has not been found to be the most appropriate or best surrogate for HuNoV, especially since the reported cultivability of MNV-1 in the laboratory.

3.2. Murine Norovirus as a Cultivable Surrogate for HuNoV

Murine norovirus (MNV-1) is a genogroup V *Norovirus* within the *Caliciviridae* family. It is a small, 28–35 nm in diameter, icosahedral, non-enveloped, single-stranded RNA virus, ~7.6 kb, with 3 ORFs (Wobus et al. 2006). MNV-1 was first isolated from severely immunocompromised

recombination-activating gene 2 (RAG2) and signal transducer and activator of transcription 1 (STAT-1) (RAG2/STAT1–/– mice) deficient mice in which it caused a systemic infection and ultimate lethality (Karst et al. 2003). MNV-1 is currently the only known norovirus that can be reproducibly propagated in cell culture using murine macrophage RAW 264.7 cells in the laboratory (Wobus et al. 2006). MNV-1 belongs in the same genus as human noroviruses with similar biochemical and genetic properties. For example, it is similarly resistant to acid and heat treatments, highly stable and persistent in the environment and shed in feces; therefore, it is considered as an appropriate surrogate for HuNoVs (Cannon et al. 2006; Wobus et al. 2006; Taube et al. 2009; Taube et al. 2013). However, MNV-1 does differ from HuNoVs in several aspects, as it does not cause the typical symptoms of gastroenteritis, such as vomiting and diarrhea, uses sialic acid as a receptor compared to the HBGAs used by HuNoVs, and also differs in pathogenesis and immunity (Karst et al. 2003; Wobus et al. 2006). Although, MNV-1 is currently being used more extensively than FCV-F9 in persistence and inactivation studies (Cannon et al. 2006; D'Souza and Su 2010; Su et al. 2010; Fraisse et al. 2011; Hirneisen et al. 2011; Tung et al. 2013), the search for the most suitable surrogate continues, along with attempts at HuNoV propagation in cell-culture systems.

3.3. Tulane Virus as a Cultivable Surrogate to Determine HuNoV Infectivity

Tulane virus (TV) (also known as monkey calicivirus) represents a new genus, *Recovirus,* within the *Caliciviridae* family. It is similar to HuNoVs in capsid size and shape, being a small, 36 nm in diameter, non-enveloped single-stranded RNA virus. Tulane virus was first reported to be isolated in stools of captive juvenile rhesus macaques (*Macaca mulatta*) at the Tulane National Primate Research Center (Farkas et al. 2008; Yu et al. 2013). Tulane virus is also reported to have the shortest RNA genome, of approximately 6.7 kb, among the known caliciviruses. It encodes 3 ORFs but lacks the typical poly (A) tail. Phylogenetic analysis of non-structural proteins of TV has indicated that it has the highest identity with HuNoVs among the known cultivable surrogates. TV virus has been successfully adapted to and propagated in monkey kidney cells (LLC-MK2 cells). In addition, TV is known to recognize the type A and B HBGAs similar to HuNoVs, suggesting antigenic similarity to HuNoVs (Farkas et al. 2010). Also, TV has been isolated from feces of animals, though without symptoms of diarrhea (Li et al. 2012c). These characteristics make TV a promising HuNoV surrogate.

TV and MNV-1 were both found to be resistant to high hydrostatic pressure treatments of 600 MPa for 2 min at different temperatures (4, 21 and 35 °C) on un-wetted (dry) blueberries; while both viruses were successfully inactivated at ≤400 MPa on wet blueberries immersed in phosphate buffered saline (Li et al. 2013). Moreover, no significant differences in heat inactivation between MNV-1 and TV have been reported, though TV showed significantly higher reductions in infectious virus titers when compared to MNV-1 after treatment at pH 2.0 and 9.0 and after 2 ppm chlorine treatments (Farkas et al. 2008;

Hirneisen and Kniel 2013). Using $TCID_{50}$ assays, titer reductions of TV were observed at pH 2.5 and 9.0, with complete inactivation at pH 10.0 and also after thermal inactivation at 63 °C for 5 min or 56 °C for 30 min, with UVC irradiation doses of 60 mJ/cm2 and with treatments of 300 ppm of free chlorine for 10 min (Tian et al. 2013). Therefore, the choice of TV as the most appropriate cultivable surrogate for determining HuNoV infectivity after inactivating treatments remains debatable.

3.4. Porcine Sapovirus as a Cultivable HuNoV Surrogate to Determine Infectivity

Porcine sapovirus belongs to the genus *Sapovirus* within the *Caliciviridae* family. The Cowden strain of porcine sapovirus is, to date, the only successfully propagated sapovirus and can be cultured in the continuous porcine kidney cell line (LLC-PK) (Flynn and Saif 1988). The presence of bile acid or intestinal content fluid filtrate obtained from uninfected gnotobiotic pigs was reported to be required for viral growth (Chang et al. 2004). Human sapoviruses are known to cause gastroenteritis in humans with similar symptoms as those caused by HuNoVs. However, similar to HuNoVs, wild-type strains currently remain uncultivable in the laboratory.

Porcine sapovirus has distinctive properties for its consideration as a suitable HuNoV surrogate. For example, it is an enteric virus genetically related to HuNoVs, the Cowden strain is cultivable in the lab, it replicates in intestinal cells of pigs, and it causes gastroenteritis in pigs, with symptoms that include diarrhea (Guo et al. 2001; Li et al. 2012c). Additionally, it is similar to HuNoVs in resistance to heat and chlorine treatment and it is stable upon exposure to pH 4–8 at room temperature for 1 h with less than a l-log reduction at pH 3 (Wang et al. 2012). Porcine sapovirus was also reported to attach to lettuce leaves at its capsid isoelectric point (pH 5.0), and remained infectious on attached lettuce after 1 week of storage at 4 °C (Wang et al. 2012). However, research is needed to determine the appropriateness of porcine sapovirus as a HuNoV surrogate since its environmental persistence and an understanding its behavior in response to various processing treatments is not known.

3.5. Virus-Like Particles as Surrogates

Human norovirus virus-like particles (VLPs) can be produced in insect, plant and mammalian cells by the expression of the virus capsid protein(s). The recombinant expression of the VP1 major capsid protein of HuNoV has been shown to result in self-assembly with the formation of empty, non-infectious virus-like particles (VLPs) (Jiang et al. 1992). The VLPs thus formed are similar in structure, morphology, antigenicity and immunogenicity to the native human noroviruses, but are safer to use and relatively easy to produce/manufacturer (Li et al. 2012c; Chen and Lai 2013). Therefore, in the absence of cell-culture based infectivity assays for HuNoVs, VLPs are studied to investigate destruction of the viral particles by various treatments/processing conditions. Results from such studies can be directly compared to receptor binding

and infectivity assays. Damage to VLPs can be determined by various methods that include ELISA binding assays, direct visualization by electron microscopy and SDS-PAGE to determine alterations to the capsid proteins (Li et al. 2012c).

HuNoV VLPs were used as models to show that the capsid of HuNoV has similar stability to MNV-1 after treatment with gamma irradiation (Feng et al. 2011). These researchers showed that gamma irradiation disrupted the structure of HuNoV VLPs and degraded the VP1 protein.

Loss of the structural integrity of virus-like particles (VLPs) as determined by transmission electron microscopy and loss of binding to histo-blood group antigen (HBGA) receptors were also used to determine the effect of high hydrostatic pressure processing (HHP) in causing disruption of the virus capsid (Lou et al. 2012). These researchers reported that HHP treatments with 500–600 MPa for 2 min (and even after a holding time of 60 min at 4 °C for each pressure treatment) did not result in any significant disruption of the structure of HuNoV VLPs as determined by transmission electron microscopy, even though the cultivable surrogates, MNV-1 and FCV-F9 were inactivated. Thus, they showed that HuNoV VLPs were quite resistant to high pressure treatments. However, HuNoV VLPs were reported to disrupt at 700, 800, and 900 MPa after 45, 15, and 2 min, respectively (Lou et al. 2012). Thus, they concluded that HuNoV VLPs, though not ideal representatives of HuNoV, could be potential alternates to determine loss in HuNoV infectivity after treatments.

Green fluorescent protein-labeled VLPs (GFP-VLPs) produced with the full-length VP2 and VP6 rotavirus capsid proteins in a baculovirus expression system were used as human rotavirus surrogates to determine their stability under different environmental conditions using flow cytometry (Caballero et al. 2004). GFP-VLPs were reported to show similar behavior when compared to infectious rotaviruses after 1 month of storage in seawater at 20 °C, but they did persist longer than infectious rotaviruses in fresh water containing 1 mg/L of free chlorine. In addition, they were reported to be more resistant to UV light irradiation than infectious rotavirus in fresh and marine water. The authors concluded that the recombinant VLP surrogates offer new options for the validation of virus inactivation or removal in actual field situations where pathogenic agents cannot be introduced (Caballero et al. 2004).

4. ANIMAL MODELS AND HUMAN FEEDING STUDIES

4.1. Animal Models

To date, considerable effort has been made towards developing a robust small-animal model for HuNoV. Such a model would further our understanding of virus infectivity after various treatments or processing technologies are

applied. An animal model for HuNoV can also be used to study immune responses to therapeutics or vaccines (Rockx et al. 2005). In a study determining the susceptibility of common marmosets, cotton top tamarins, cynomolgus, and rhesus macaques to HuNoV infection following oral inoculation, rhesus macaques were found to be susceptible to Norwalk virus infection as one animal shed virus for a long period of time and developed Norwalk virus specific IgM and IgG responses (Rockx et al. 2005). These researchers also reported that low-level replication could have occurred in common marmosets and cotton top tamarins but not in cynomolgus macaques, based on short-term viral shedding without any clinical symptoms or antibody responses.

Another study investigated the feasibility of using chimpanzees as an animal model for HuNoV (Bok et al. 2011). When these researchers intravenously inoculated seronegative chimpanzees with Norwalk virus, these animals were reported to not show any clinical signs of gastroenteritis, though the onset and duration of viral fecal shedding and serum antibody responses were similar to that in humans after infection. These authors also detected Norwalk virus RNA in intestinal and liver biopsies, along with the simultaneous detection of viral shedding in stool. The authors also reported that chimpanzees inoculated intramuscularly with GI VLPs (to determine the efficacy of VLPs as vaccines) were protected from NV infection when challenged 2 and 18 months after vaccination. The authors thus concluded that chimpanzees could be viable animal models for use in HuNoV replication and immunity studies (Bok et al. 2011).

Gnotobiotic pigs expressing A type or H type-1 HBGAs on their enterocytes were used as animal models to determine the effect of oral treatment with the commonly used cholesterol-lowering medication, simvastatin, which was found to increase the replication of HuNoV in an *in vitro* system (Jung et al. 2012). These researchers showed that significantly early onset and longer duration of HuNoV fecal shedding with high viral titers in feces were obtained in simvastatin-treated pigs. This simvastatin-induced increase in HuNoV shedding was reported to be decreased in the HuNoV-inoculated pigs that were pre-treated or treated with human interferon (IFN)-α, which is known to decrease HuNoV infectivity (Jung et al. 2012). The authors concluded that gnotobiotic pigs can be a useful model to determine the efficacy of antivirals against HuNoV and/or inactivation of HuNoVs. Gnotobiotic calves have also been suggested for use as animal models for HuNoV, though the cost of using gnotobiotic animals is relatively high (Li et al. 2012c).

It has also been shown by Taube et al. (2013) that when humanized and non-humanized BALB/c recombination activation gene (Rag)-common gamma chain (γc)-deficient mice were intra-peritoneally challenged with pooled stool samples from HuNoV positive patients, increased GII.4 viral load was obtained. In addition, structural and non-structural proteins were detected in their spleen and livers, suggesting HuNoV replication. However, immunocompetent wild-type BALB/c mice did not appear to be infected with HuNoV after challenge (Taube et al. 2013). These researchers thus described the first genetically manipulated small-animal model (with mice) for studying HuNoV infection.

4.2. Human Challenge Studies

Human challenge studies, though most suitable to determine inactivation of virus particles after processing treatments, have many challenges that include high cost, and a limited number of researchers that can have the facilities, resources or capabilities for conducting challenge studies with human subjects. Though, Richards (2013) suggests that the cost and risk associated with HuNoV infection is much higher than the perceived cost of few volunteer studies. He therefore recommends/proposes a shift from surrogate use to volunteer studies for scientific validation of processing technologies to improve safety of foods (Richards 2013). A randomized, double-blinded clinical feeding study was used to determine the inactivation of HuNoV GI.1 (10^4 genomic equivalent copies) in spiked oysters by high hydrostatic pressure processing (HPP) using 44-healthy subjects. These subjects received either virus treated with 400 MPa HPP at 25 °C, 600 MPa at 6 °C, or 400 MPa at 6 °C for 5 min or untreated virus included as a control (Leon et al. 2011). These researchers showed that 600 MPa for 5 min, but not 400 MPa (at 6 °C or 25 °C) was required to completely inactivate HuNoV in seeded oysters without causing human infection.

5. SUMMARY AND CONCLUSIONS

There is on-going research in the development of alternate approaches to determine the infectivity of viruses after various inactivating treatments and to determine their persistence in the environment, which is considered a major bottleneck in the determining appropriate control strategies. As noted by Knight et al. (2013), long RT-PCR could potentially differentiate between intact and degraded RNA in survival and persistence studies after inactivation treatments, though the assay sensitivity for food samples may be a limitation. While the search for suitable host systems for the culture of currently non-cultivable foodborne viruses continues, alternate approaches to estimating virus infectivity are under development and evaluation. However, results from these studies must still be interpreted with caution, since these methodologies do not seem to perform identically for all types of inactivating treatments. There are still many issues and challenges with these new approaches that need to be overcome. Such issues include, their application with virus extraction procedures for complex food matrices and environmental samples, additional processing time constraints, assay detection sensitivities and costs, compatibility with food matrices and possible PCR inhibitors and finally validation by laboratories for routine use (Hamza et al. 2011). In addition, appropriate controls (both extraction and amplification controls) need to be used in designing these infectious diagnostic assays and experiments should be carefully planned for assay robustness. As described by researchers before, the effect of various procedures for virus extraction from food and environmental

matrices on virus capsid integrity needs to be understood. Therefore, a standard method that defines "infectivity" for HuNoVs still remains complicated (Knight et al. 2013). While various alternate approaches now available to estimate virus infectivity in the absence of cell-culture based systems have been summarized in this chapter, their direct correlation with the appropriate cell-culture based infectivity methods would be most appropriate for HuNoVs and wild-type HAV. Hence, there is a crucial need to find suitable host systems to cultivate the HuNoVs and wild-type HAV in the laboratory. Jones et al. (2014) demonstrated that the presence of HBGA-expressing enteric bacteria was required for the infection of B cells by HuNoV and validation of this replication model is still needed by other laboratories. Until then, a general consensus on the best strategies to determine infectivity of non-culturable foodborne viruses needs to be reached among researchers worldwide.

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CHAPTER 13

Survival of Enteric Viruses in the Environment and Food

Gloria Sánchez and Albert Bosch

1. INTRODUCTION

Enteric viruses are those human viruses that are primarily transmitted by the fecal-oral route, either by person-to-person contact or by ingestion of contaminated food or water. The importance of viral foodborne diseases is increasingly being recognized, and several international organizations have found that there is an upward trend in their incidence. Consequently, there is a growing concern over human exposure to enteric viruses through contaminated food products. Data on viral foodborne diseases are still fragmented, and epidemiological studies have focused either on particular countries or on particular pathogens. In the last decade, epidemiological reports indicate that enteric viruses, in particular human noroviruses (NoV), which cause acute gastroenteritis, and hepatitis A virus (HAV), are the leading cause of foodborne illness in developed countries (Koopmans and Duizer 2004; EFSA 2015). Other enteric viruses, including rotaviruses, sapoviruses, astroviruses and hepatitis E virus (HEV), are not frequent causes of foodborne disease but can occasionally be transmitted by contaminated foods.

In numerous NoV or HAV cases, the vehicle(s) of virus spread and food contamination remains unidentified. Much epidemiological evidence suggests that infected food handlers and contaminated food-contact surfaces may play an important role in food contamination. Food may become contaminated with enteric viruses either by fecal contamination, cross contamination from another food product, or by an infected food handler. While consumption of ready-to-eat foods contaminated by infected food handlers remains the major risk factor for viral foodborne outbreaks, many types of food products are being recognized as vehicles of viruses in causing gastroenteritis or hepatitis A outbreaks (Table 13.1).

In the EU, foodborne viruses (mainly human NoVs) were identified to be the most frequently detected causative agents of foodborne outbreaks in 2014, accounting for 20.14 % of the reported outbreaks (EFSA 2015). In 2011, the Centers for Disease Control and Prevention (CDC) issued new figures for the incidence of foodborne illness, estimating that about 5.5 million people in the USA suffer from viral foodborne illnesses each year, resulting in 15,300 hospi-

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talizations and 156 deaths. Moreover, the cost of foodborne illness in the U.S.A. is now estimated to be around $3000 million a year according to a study conducted by the Ohio State University (Scharff 2012).

As a reflection of the seriousness of viral foodborne outbreaks, extensive attention has been given to them by national and international organizations over the last 10 years. Examples of which include: the report of the Advisory Committee on the Microbiological Safety of Food, the recently proposed guidelines for the application of food hygiene to the control of viruses for Codex Alimentarius (CX/FH/10/42/5), the scientific opinion of the European Food Safety Authority (EFSA) (http://www.efsa.europa.eu/fr/efsajournal/pub/2190), and the expert advice on foodborne viruses for Codex Alimentarius (www.who.int/foodsafety/publications/micro/mra13/en/index.html). This latter document concluded, among other considerations, that prevention and control measures should be considered for enteric viruses in bivalve molluscan shellfish, fresh produce, or prepared foods.

Virus persistence Because viruses outside their hosts are inert particles, their chances of transmission from host to host are greatly dependent on the degree of their robustness, which allows them to remain infectious during the various conditions they encounter in the environment and foods. Numerous physical, chemical, and biological factors influence virus persistence in the environment (Table 13.2). Some of the primary factors affecting the survival of viruses in liquid environmental matrices are temperature, ionic strength, chemical constituents, microbial antagonism, the sorption status of the virus, and the type of virus. Considerable differences have been observed in the survival of enteric viruses in different types of environmental and food samples. Different behaviors and inactivation rates have been observed not only among viruses of different families and genera, but also among viruses of the same family, genus, and even among similar types or strains of virus. Among the chemical constituents of liquid or semisolid (feces, human night soil, biosolids, and animal manures, etc.) environmental matrices, the amount and type of organic matter and specific antiviral chemicals (such as ammonia at elevated pH levels or natural antimicrobial compounds of fruits) play a role in virus stability. Of the physical factors influencing virus persistence in liquid media, temperature, sunlight, and virus association with solids are among the most important. Soil moisture, temperature, sunlight, and other soil characteristics may influence the persistence of viruses in soil. On inanimate surfaces (or fomites), the most important factors that affect virus stability are the type of virus and surface, relative humidity, moisture content, temperature, composition of the suspending medium, light exposure, and presence of antiviral chemical or biological agents. Most of these factors are also relevant for the ability of viruses to persist in aerosolized droplets, together with the moisture content and the size of the aerosol particles, and the air quality.

Understanding food and environmental virus stability, and elucidating the factors that affect it, may shed some light on the potential public health risk associated with these viruses and at the same time provide tools to interrupt the chain of fecal-oral transmission. In this chapter, only studies involving the

Table 13.1 Selected large outbreaks (over 100 cases) of hepatitis A virus and norovirus occurring in the last 10 years

Year	*Location*	*Implicated food*	*Responsible virus*	*Raw material origin*	*Number of cases*	*Reference*
2012–2013	Nordic European countries	Frozen strawberries	HAV		103	Nordic outbreak investigation team 2013
2012	Germany	Frozen strawberries	NoV	China	>11,000	http://www.bfr.bund.de/en/norovirus_outbreak_2012-186684.html
2011	US	Pomegranate seeds	HAV	Turkey	162	http://www.cdc.gov/hepatitis/Outbreaks/2013/A1b-03-31/index.html
2010	Denmark	Lettuce	NoV	France	260	Ethelberg et al. (2010)
2009–2010	Australia, The Netherlands, France	Sun dried tomatoes	HAV	Turkey	308	Gallot et al. (2011), Petrignani et al. (2010)
2008	China	Bottled water[a]	HAV	China	269	http://www.china.org.cn/government/local_governments/2008-04/24/content_15007889.htm
2007	France	Oysters	HAV		111	Guillois-Bécel et al. (2009)
2005	Denmark	Frozen raspberries	NoV	Poland	>1000	Falkenhorst et al. (2005)
2004	Belgium	Raw beef[b]	HAV		269	Robesyn et al. (2009)
2004	Egypt	Orange juice[c]	HAV		351	Frank et al. (2007)
2003–2004	Singapur	Oysters[d]	NoV		305	Ng et al. (2005)
2003	USA	Green onions	HAV	Mexico	601, 3 deaths	Wheeler et al. (2005)

[a]Suspected contamination of the water source with runoff from melting of heavy snowfall
[b]Food handler contamination
[c]Poor hygiene during processing
[d]Frozen and served thawed

Table 13.2 Factors affecting virus persistence in the environment

Factor	*Effect*
Physical	
Heat	Inactivation is directly proportional to temperature
Light	Light, especially its UV component, is germicidal
Desiccation or drying	Usually inactivation increases at lower relative humidity
Aggregation/adsorption	Protection from inactivation
Pressure	High pressure induces inactivation
Chemical	
pH	Worst stability at extreme pH values
Salinity	Increased salt concentrations are virucidal
Ammonia	Virucidal
Inorganic ions	Some (e.g. Ag, Pt, Pd, Rh) are virucidal
Organic matter	Dissolved, colloidal, and solid organic matter protect from inactivation
Enzymes	Proteases and nucleases contribute to inactivation
Biological	
Microbial activity	Contributes to inactivation
Protozoal predation	Contributes to removal/death
Biofilms	Adsorption to biofilms protects from inactivation, while microbial activity in biofilms may be virucidal
Type of virus	Stability varies according to the strain and type of virus

persistence of enteric viruses in the absence of any deliberately applied inactivation process are reviewed. Neither work on virus disinfection nor studies conducted with potential indicators, such as bacteriophages, are considered.

2. METHODS TO STUDY VIRUS PERSISTENCE IN FOOD AND THE ENVIRONMENT

Most studies to determine the potential of enteric viruses to persist in food matrices or in the environment have been performed by artificially adding a known amount of virus to a given sample, determining the reduction in the infectious titer after subjecting the spiked sample to designated conditions, and applying statistical procedures to determine the significance of virus decay. Obviously, this implies the use of virus strains that may be propagated in cell cultures and enumerated through infectivity, thus greatly restricting the range of viruses that are able to be included in these studies.

This is extremely relevant for NoV, since although attempts to culture them have been made (Guix et al. 2007; Straub et al. 2007; Papafragkou et al. 2013; Jones et al. 2014), research with human NoV has been hampered by the lack of suitable laboratory animals and the inability to propagate the virus in vitro. Consequently, the use of surrogates including feline calicivirus (FCV), murine norovirus (MNV), and Tulane virus (TV) is being extensively used to evaluate the NoV persistence in the environment and different food matrices.

Moreover, in the last decade molecular detection approaches such as RT-PCR or real-time quantitative RT-PCR (RT-qPCR) are normally employed for fastidious virus analysis. However, these techniques are unable to differentiate between infectious and non-infectious particles and, therefore, unsuitable for virus persistence studies. A promising approach to avoid this drawback relies on the use of nucleic acid intercalating dyes such as propidium monoazide (PMA) or ethidium monoazide (EMA) as a sample pretreatment previous to the RT-qPCR. So far, PMA combined with RT-qPCR has successfully been applied to discriminate between infectious and heat-treated non-infectious viruses e.g., poliovirus, coxsackievirus, echovirus and HAV (Parshionikar et al. 2010; Sánchez et al. 2012; Coudray-Meunier et al. 2013). Moreover, EMA has also been used to distinguish between thermally inactivated MNV and poliovirus suspensions (Kim and Ko 2012).

Other alternative strategy to increase the likelihood of detecting intact and potentially infectious viruses is to pretreat the virions with nucleases and/or proteolytic enzymes prior to nucleic acid extraction, amplification, and detection, thereby eliminating the detection of free nucleic acids or nucleic acids associated with damaged, inactivated virions (Nuanualsuwan and Cliver 2003).

Some other health significant enteric viruses, such as rotavirus, astrovirus, and enteric adenovirus, replicate poorly in cell cultures; yet their persistence may be evaluated by integrated cell culture RT-PCR assays (Pintó et al. 1995; Reynolds et al. 1996; Abad et al. 1997; Reynolds et al. 2001). For this purpose, cells supporting the propagation of a wide variety of enteric viruses, such as CaCo-2 (colonic carcinoma) or PLC/PRF/5 cells (human liver hepatoma), have been used as an *in vivo* amplification step prior to molecular amplification (Grabow et al. 1993; Pintó et al. 1994).

Another issue to be considered from an experimental point of view is how the survival experiments are designed. Most studies are performed by artificially adding a known amount of virus to a food sample without considering all the mechanisms of attachment involved. For instance, enteric viruses bind to food products by a variety of mechanisms, including ionic and hydrophobic interactions, van der Waals forces, interaction with receptors (e.g. NoV binding to carbohydrates) and uptake into bivalve mollusk and vegetable tissues, which may have an impact on its survival on these food items.

Moreover, most of the methods for virus detection in food include a key elution step to release the viruses from the food surface, because it is assumed that naturally contaminated samples carry virus particles only on the surface. However, enteric viruses can also attach to the leaf surface and internalize

through stoma and cuts on the leaf during direct contact with contaminated water (Wei et al. 2011). Furthermore, a new mechanism of HAV contamination of green onions was proposed by Chancellor et al. 2006. In this study HAV particles were found trapped inside growing green onions taken up intracellularly through the roots, even though survival of the virus was not evaluated. This mechanism has also been described for NoV and NoV surrogates such as MNV, Tulane virus, porcine sapovirus and canine calicivirus on lettuce (Dicaprio et al. 2012; Esseili et al. 2012; Urbanucci et al. 2009; Wei et al. 2011) and green onions and spinach (Hirneisen and Kniel 2013). This mechanism will definitely change future approaches for the detection of viruses in vegetables, as well as the design of survival experiments in vegetables.

3. VIRUS PERSISTENCE IN THE ENVIRONMENT

Persistence or stability are the terms of choice to describe the capacity of a given virus to retain its infectivity in a given scenario. One critical question in environmental virology is whether or not viruses can persist long enough, and in high enough concentrations in the environment, to cause disease in individuals who are in contact with polluted recreational water, soil, fomites, or contaminated hands.

Some enteric virus infections follow a seasonal pattern, whereas others fail to do so. In regions with temperate climates, infections due to enteroviruses generally reach a peak in summer and early fall. On the contrary, rotavirus, NoV, and astrovirus infections occur mainly during the cooler months (McNulty 1978; Mounts et al. 2000; Guix et al. 2002), although seasonal and non-seasonal distributions of rotavirus in sewage have been described (Hejkal et al. 1984; Bosch et al. 1988). On the other hand, cases of hepatitis A do not show a clear seasonal pattern (Lemon 1985), whereas enteric adenovirus infections are reported to peak in midsummer (Wadell et al. 1989). These data suggest that temperature, and probably relative humidity, may be meaningful in the seasonal distribution of outbreaks of certain human enteric viruses (Enright 1954), due to the influence of these factors on virus persistence.

3.1. Virus Persistence in Environmental Waters

The survival of viruses in environmental waters has been extensively reviewed (reviewed by Rzezutka and Cook 2004). As previously mentioned, the most relevant factors affecting virus survival in the water environment are: temperature, virus association with solids, exposure to UV, and the presence of microbiota. The effect of temperature on viral persistence in water may be due to several mechanisms including protein denaturation, RNA damage, and influence on microbial or enzymatic activity (Dimmock 1967; Melnick and Gerba 1980; Deng and Cliver 1995). Early studies pointed to damage to virion proteins as the primary target for viral inactivation at high

temperatures, although damage to both protein and RNA occurs at all temperatures (Dimmock 1967). Even though all viruses persist better at lower temperatures than at higher temperatures, some enteric viruses, such as HAV and parvovirus, do exhibit higher thermal resistance than other viruses.

As mentioned earlier in this chapter, virus adsorption to particulate material increases the persistence of enteric viruses in the water environment (Gerba and Schaiberger 1975; La Belle et al. 1980; Rao et al. 1984; Sobsey et al. 1988), although differences have been observed among study locations (La Belle et al. 1980; Sano et al. 2011; Pérez-Sautu et al. 2012). The increased virus survival in the presence of sediment has important implications in the marine environment, because fecal contamination of coastal areas results in contamination of shellfish harvesting areas, accumulation of solid-associated viruses in sediments with sediments acting as virus reservoirs, and finally accumulation of viruses in shellfish. Additionally, virus uptake by molluskan bivalves is enhanced by the presence of particulate material (Landry et al. 1983).

Although self-purification processes are reported to be more pronounced in seawater than in river water (Matossian and Garabedian 1967; Gironés et al. 1989), the effect of salinity on virus stability is variable. Thus, many studies have reported enhanced removal of virus infectivity in saline solution compared with distilled water (Dimmock 1967; Salo and Cliver 1976), whereas others report no significant effect of salinity on virus persistence (Lo et al. 1976; Fujioka et al. 1980). In any case, the self-depuration capacity of water is finite.

Several observations demonstrate the potential involvement of native aquatic microorganisms in the inactivation of viruses, particularly in marine habitats. However, data on the successful isolation of microorganisms with virucidal properties are scarce (Fujioka et al. 1980; Gironés et al. 1990; Bosch et al. 1993). Additionally, the ability of bacteria to inactivate viruses is usually lost while subculturing the microorganisms in the laboratory (Gunderson et al. 1968; Katzenelson 1978), although in a few studies, such bacteria could be subcultured for more than 1 year without losing their antiviral activity (Gironés et al. 1990; Bosch et al. 1993). In some studies, the virucidal agents in the tested waters could not be separated from the microorganisms (Shuval et al. 1971; Denis et al. 1977; Fujioka et al. 1980; Ward et al. 1986; Gironés et al. 1990), whereas in others the virucidal activity could be separated from the bacteria (Matossian and Garabedian 1967; O'Brien and Newman 1977; Toranzo et al. 1983; Bosch et al. 1993). The antiviral activity seems to be based on proteolytic bacterial enzymes that inactivate virus particles in water by cleavage of viral proteins, thus exposing the viral RNA to nuclease digestion (Toranzo et al. 1983; Gironés et al. 1990; Bosch et al. 1993).

It seems reasonable to assume that environmental factors and the compositional makeup of a given type of water may be substantially different from one geographical location to another, which implies that different data of virus persistence are produced when the same viral strain is suspended in water sampled from different sites (Bosch et al. 1993). Furthermore, it is highly likely that natural waters, particularly in the marine environment, contain a variety

of potential antiviral factors, and that the antiviral action observed is generally the expression of the most dominant factor(s) present in any given water source.

3.2. Virus Persistence in Soil

Diseases associated with soil have been categorized according to the origin of the etiological agent as follows (Weissman et al. 1976; Santamaría and Toranzos 2003): (a) soil-associated diseases that are caused by opportunistic or emerging pathogens belonging to the normal soil microbiota; (b) soil related diseases that result in intoxication from the ingestion of food contaminated with entero- or neurotoxins; (c) soil-based diseases caused by pathogens indigenous to soil; and (d) soil-borne diseases caused by enteric pathogens that get into soil by means of human or animal excreta. In this latter category are included viruses transmitted through the fecal-oral route.

The transport of viruses through soil to groundwater and then to the community has been a topic of great concern. Many epidemics of infectious diseases have been attributed to the consumption of contaminated groundwater, casting soil as a vector and source of important human disease agents (Asano and Cotruvo 2004; Craun et al. 2010). There is a concern about a possible increase in soil-borne diseases in human population, given the land disposal practices of sewage and sewage sludge. In developing countries, untreated domestic wastewater is used in agricultural irrigation, presenting a high risk to farm workers and to consumers of food products irrigated with wastewater (Strauss 1994). In spite of the clear public health implications of the occurrence and survival of viruses in the soil compartment, studies on the fate of viruses in soil are scarce due to the complexity of the methodologies for virus extraction from soil.

The most relevant factors controlling virus transport through soil are soil type, water saturation state, pH, conductivity of the percolating water, and soluble organic matter (Table 13.2). The type of soil has a great influence on the level of viral transport. Fine-textured soils tend to absorb viruses more readily than coarsely textured soils. As a general rule, sandy soils are relatively poor adsorbents of enteric viruses, whereas soils with clay content of 30–100 % are excellent adsorbents (Sobsey et al. 1980). In consequence, viral adsorption increases with increasing clay mineral content (Gerba et al. 1981). The high adsorptive properties of a clay soil will prevent virus transport to another matrix, such as groundwater, whereas coarse soil will not.

Microbial movement in soils is also greatly dependent on the water saturation state. When the soil is saturated, all pores are filled with water, which allows faster virus transport through the soil because virus contact with the soil has been diminished. When the flow is unsaturated, the viruses are in closer contact with the soil, thus promoting virus adsorption to the soil (Santamaría and Toranzos 2003). Goyal and Gerba (1979) considered soil pH as the single most important factor influencing viral adsorption, although the combined effect of organic matter and clay content, and cation-exchange capacity, could

surpass the sole soil pH effect. At ambient conditions, viruses are usually negatively charged, thus being attracted to and entrapped by positively charged material in soil (Sobsey et al. 1980). In neutral and alkaline soil situations, viruses will not bind to any particulate matter and will be allowed to move freely in soil. There are, however, many exceptions to these general rules. Virus absorption to soil is also affected by cation concentrations. Cations favor virus adsorption to soil by reducing their repulsive forces. Sewage wastes provide an environment that enhances virus retention to soil, while this retention would be low in distilled water. As a matter of fact, distilled water may actually lead to the elution of viruses from soils, favoring virus mobilization and transport through soil. On the other hand, soluble organic matter will compete with the virus for soil adsorption sites. Likewise, humic and fulvic acids will also compete with the virus and will reduce the level of adsorption of viruses to the soil (Sobsey and Hickey 1985). Wei et al. (2010) investigated murine norovirus (MNV) and HAV stability on three types of differently treated biosolids at 20 and 4 °C and they reported that both viruses were inactivated rapidly in alkaline pH biosolids.

3.3. Virus Persistence in Aerosols

Aerosols are an important means of virus transmission in humans. Various authors have reported the isolation of enteric viruses from aerosols produced by sludge-treatment plants (Fannin et al. 1985; Fattal and Shuval 1989; Pfirrmann and Bossche 1994; Alvarez et al. 1995; Carducci et al. 1999). The presence of microorganisms in aerosols generated from wastewater-treatment processes or in treated wastewater for agricultural irrigation is a potential danger to human health (Teltsch et al. 1980; Alvarez et al. 1995). In hospitals, aerosolization of vomit was reported to be of major importance in the transmission of NoV infection during outbreaks, while cleaning vomit or feces from patients did not significantly increase the risk of developing gastroenteritis (Chadwick and McCann 1994). Members of the *Caliciviridae* family have been reported to be fairly stable in aerosols (Donaldson and Ferris 1976). The most important factors affecting the stability of viruses in the aerosol state are temperature, pH, relative humidity, moisture content, size of the aerosol particle, composition of the suspending medium, sunlight exposure, air quality, and virus type.

The basis of virus inactivation in aerosols is poorly understood, although mechanisms for bacteriophage inactivation in aerosols have been proposed (Trouwborst et al. 1974). At high relative humidity, surface alteration of the virion has been reported, whereas at low relative humidity virus inactivation appears to be mediated by the removal of structural water molecules. Relative humidity seems to confer a protective effect on aerosolized non-enveloped virus particles. Thus, poliovirus was more stable in aerosol at 22 °C at high relative humidity than at low relative humidity (Harper 1961). Picornavirus infectious RNA may be detected at all humidity levels, suggesting that virus inactivation is caused by virion capsid damage (Akers and Hatch 1968). High

relative humidity and low temperature enhance the persistence of bovine rotavirus in aerosols (Moe and Harper 1983; Ijaz et al. 1985), although simian rotavirus SA11 survival in aerosols seems to be the best at intermediate relative humidity levels (Sattar et al. 1984). In any case, human, simian, and calf rotavirus strains may be detected in aerosols after as long as 10 days (Moe and Harper 1983; Sattar et al. 1984; Ijaz et al. 1985), although discrepancies, probably due to methodological differences, are found among these studies. Aerosolized adenovirus particles also show increased persistence at high relative humidity and low temperature (Miller and Artenstein 1967; Elazhary and Derbyshire 1979).

Contrarily to non-enveloped viruses, viruses with an outer lipid envelope seem to be more stable at lower relative humidity (Hemmes et al. 1960). After 6 days at 20 °C and 50 % relative humidity, infectious human coronavirus particles could be recovered in aerosols (Ijaz et al. 1985). Virus infectivity in aerosols is also affected by solutes in the suspending media used for aerosolization. Addition of salts and proteins in the suspending media provides a protective effect against dehydration and thermal inactivation of aerosolized picornaviruses (McGeady et al. 1979; Reagan et al. 1981) and may also influence the rehydration rate during sample re-humidification prior to the infectivity assay (Benbough 1969).

3.4. Virus Persistence on Fomites

Outbreaks of acute gastroenteritis and hepatitis are a matter of concern in institutions such as, hospitals, daycare centers, nurseries, schools, restaurants, and military quarters. Many of these outbreaks have been suspected to be caused by vehicular transmission of agents through contaminated environmental surfaces (fomites). Stools from patients with diarrhea or hepatitis contain a very high number of the causative virus, and a single vomiting episode of an individual suffering from NoV gastroenteritis may expel 3×10^7 virus particles, all of which are able to contaminate fomites (Cheesbrough et al. 1997; Green et al. 1998).

It has been demonstrated that human enteric viruses are able to survive on several types of materials commonly found in institutions and domestic environments long enough to represent a source for secondary transmission of disease (Hendley et al. 1973; Sattar et al. 1986, 1987; Ansari et al. 1988; Mbithi et al. 1991; Abad et al. 1994, 2001). The stability of health-significant human enteric viruses has been investigated on various non-porous (aluminum, china, glazed tile, glass, latex, plastic, polystyrene and stainless steel) and porous (cloth, different types of papers and cotton cloth) surfaces (Sattar et al. 1986; Abad et al. 1994, 2001; Boone and Gerba 2007). As a general conclusion, when dried on environmental fomites, HAV and rotavirus are more resistant to inactivation than enteric adenovirus, astrovirus, and poliovirus.

The higher stability of HAV in comparison with poliovirus, both of which belong to the *Picornaviridae* family, is due to the inherently more stable

molecular structure of HAV capsid, concordant with the special codon usage described for this virus (Sánchez et al. 2003). In fact, it appears undeniable that poliovirus, which has been extensively employed as a model to elucidate enteric virus behavior in many scenarios, may fail to provide an adequate indication of the persistence of other human enteric viruses, such as HAV, astrovirus, or rotavirus, dried on fomites (Sobsey et al. 1988; Mbithi et al. 1991; Abad et al. 1994, 2001).

The resistance to desiccation appears to be of major significance in determining the ability of a virus strain to survive on fomites. A pronounced loss in virus titer at this stage dramatically reduces the chances of subsequent virus persistence. On the contrary, viruses involved in outbreaks probably transmitted through faecally contaminated environmental surfaces (i.e., HAV, NoV, rotavirus, or astrovirus) show little decay at the desiccation step (Mahl and Sadler 1975; Keswick et al. 1983; Sattar et al. 1986; Sobsey et al. 1988; Abad et al. 1994, 2001).

In spite of the experimental data on virus persistence on environmental surfaces, it is generally very difficult to determine whether, and to what extent, fomites play a role in the spread of infectious agents. Keswick et al. (1983) have suggested that the prevalence of asymptomatic infections in daycare facilities may make contaminated surfaces in these environments a reservoir of infection for previously uninfected inmate children and their family contacts.

Because the fecal-oral route is the common means of enteric virus transmission, it seems reasonable to evaluate the effect of fecal material on the persistence of virus on fecally contaminated fomites. Again, data on the protective effect of feces on viruses are contradictory; fecal matter appears to affect the survival of enteric viruses in opposite ways, depending on the type of surface and the virus strain (Keswick et al. 1983; Sobsey et al. 1988; Abad et al. 1994).

Survival of NoV on fomites has been investigated by using surrogates or using molecular techniques. Studies using NoV surrogates are more abundant. Clay et al. (2006) investigated FCV survival on computer mouse, keyboard keys, telephone wire, telephone receiver, telephone buttons, and brass disks representing faucets and door handle surfaces. This study concluded that survival of FCV varied with fomite type. FCV was still infectious for up to 3 days on telephone buttons and receivers, for 1 or 2 days on computer mouse, and for 8–12 h on keyboard keys and brass. Mattison et al. (2007) also used FCV to investigated NoV survival on stainless steel. Temperature substantially affects the survival of FCV, which is able to persist for long periods of time dried onto glass coverslips with log reductions of 4.75 after 2 months and 3 weeks, at 4 °C and room temperature, respectively (Doultree et al. 1999). The authors suggested that the effect of temperature on FCV stability may reflect the greater prevalence of NoV infections in cooler seasons (Lopman et al. 2003).

Cannon et al. (2006) reported long-term persistence of FCV and MNV suspended in a fecal matrix and inoculated onto stainless steel coupons at 4 °C, but at room temperature MNV was more stable than FCV. Recently, MNV

was used as a NoV surrogate to investigate survival on food contact surfaces. MNV infectivity on stainless steel rapidly decreased by more than 2 log, and a complete loss of infectivity was reported at day 30 (Takahashi et al. 2011). Additionally, they also showed that the presence of food residues increased the survival of MNV, whereas only 1.4 log reduction of infectivity was reported at day 30.

D'Souza et al. (2006) investigated the stability of NoV, NoV RNA and FCV on stainless steel, formica and ceramic coupons. NoV and FCV were detected on all 3 surfaces up to 7 days post inoculation. NoV RNA was not detected beyond 24 h on stainless steel. Moreover, in this study, stainless steel coupons were inoculated with NoV or FCV and allowed to dry after which lettuce leaves were exposed to the surface of the coupons at various contact pressures. Results showed that transfer of both NoV and FCV from stainless steel surfaces to lettuce occurred easily. Recently Lopez et al. (2013) examined the effect of low and high relative humidity on fomite-to-finger transfer efficiency of poliovirus from several common fomites, showing that transfer efficiencies were greater under high relative humidity for both porous and nonporous surfaces. Gloves also may serve as a source of virus second transmission, enteric viruses could be transferred in an infectious state from gloves to other surfaces or food and vice versa (Verhaelen et al. 2013).

3.5. Virus Persistence on Hands

Strong evidence indicates that virus-contaminated hands play a major role in the spread of enteric viruses, particularly in institutional settings and during food preparation.

Contaminated human hands can transfer the virus to inanimate objects or food products, which may then spread the virus to susceptible persons (Hendley et al. 1973; Ansari et al. 1988; Mbithi et al. 1992; Bidawid et al. 2001a; Tuladhar et al. 2013). It was ascertained in these studies that rotavirus and HAV could retain infectivity for several hours on skin and could be transferred in an infectious state from fingertips to other surfaces and vice versa. For norovirus, MNV infectivity transfer from finger pads to stainless steel ranged from $13 \pm 16\,\%$, whereas similar results were found for NoV GI.4 and GII.4 transfers measured in PCR units.

Enteric virus transfer between hands was apparently influenced by moisture. Moisture would mediate suspension of virus particles and facilitate their movement between touching surfaces; drying would reduce this effect. Laboratory studies have shown that viruses persist better in the environment at high relative humidity and at low temperatures (Sattar et al. 1984; Sobsey et al. 1988; Abad et al. 1994; Bidawid et al. 2001a). However, as mentioned above, data on the effect of relative humidity on enteric virus survival is contradictory. These reported differences, particularly affecting rotavirus persistence, are difficult to explain but may be due to differences in the methodologies employed in these studies.

4. STABILITY OF ENTERIC VIRUS IN FOOD PRODUCTS

The most important factors affecting the stability of viruses in food products are virus type, temperature, pH, relative humidity, moisture content, sunlight exposure and type of food. This latter factor may have a great impact depending on the type of surface, for instance the presence of crevices and hair-like projections in berries may shield the viruses against environmental modifications or the presence of natural antiviral compounds in the food itself.

4.1. Stability of Enteric Viruses on Chilled Products

In minimally processed fruits and vegetables, chilled storage temperatures (2–11 °C) typically retard respiration, senescence, product browning, moisture loss, and microbial growth, but may contribute to the survival and transmission of enteric viruses (Seymour and Appleton 2001; Rzezutka and Cook 2004). A variety of enteric viruses have been examined for the effects of chilled temperature on their survival in a range of food matrices (reviewed by Baert et al. 2009) (Table 13.3). Most of the studies found that enteric viruses remained infectious for periods exceeding the shelf-life of products (Table 13.3). On vegetables, Croci et al. (2002) evaluated HAV survival on lettuce, carrots and fennel, reporting complete inactivation of HAV by day 4 and 7 for carrots and fennel, respectively. On lettuce a slight decrease was observed over time. Sun et al. (2012) recently reported that HAV survived more than 20 days during storage at 3–10 °C on surface inoculated green onions. Shieh et al. (2009) investigated the survival of HAV on fresh spinach leaves in moisture- and gas-permeable packages that were stored at 5.4 °C for up to 42 days, reporting only a 1 log reduction of HAV infectivity over 4 weeks of storage. In shellfish, HAV inoculated in commercially prepared marinated mussels showed a 1.7 log reduction of infectivity after 4 weeks of storage at 4 °C (Hewitt and Greening 2004).

The stability of HAV and PV inoculated in bottled water was studied at 4 °C (Biziagos et al. 1988). Infectious HAV and PV were detected after 1 year of storage, with less than 1 log reduction. This study also reported that HAV stability was dependent on the proteinaceous concentration added to the water.

Attempts to evaluate stability of human NoV in food products have been performed by using molecular techniques alone or combined with pretreatments to assess infectivity. Mormann et al. (2010) reported no reductions on NoV titers after RNase pretreatment during cooling for lettuce (5 days, 11 °C), apples (7 days, 11 °C) and mincemeat (2 days, 6 °C). Lamhoujeb et al. (2008) demonstrated that NoV survived for at least 10 days on refrigerated lettuce and turkey. In mussels no reduction on NoV titers were reported after 4 weeks of storage at 4 °C (Hewitt and Greening 2004).

Several studies have also estimated NoV stability by using surrogates. Mattison et al. (2007) investigated the survival of FCV on lettuce and straw-

Table 13.3 Survival of NoV and HAV in artificially inoculated foodstuffs during refrigeration storage

	Virus	*Food product*	*Process*	*Log reduction*	*Reference*
Refrigeration	NoV	Apple	7 days at 11 °C	0.2 (±0.76)	Mormann et al. (2010)
		Lettuce	5 days at 11 °C	0.1 (±0.15)	Mormann et al. (2010)
			10 days at 7 °C	1.78	Lamhoujeb et al. (2008)
		Mincemeat	2 days at 6 °C	0.0 (±0.61)	Mormann et al. (2010)
		Marinated mussels (pH 3.75)	28 days at 4 °C	0.0	Hewitt and Greening (2004)
		Raspberries	7 days at 4 °C	0.0	Verhaelen et al. (2012)
			7 days at 10 °C	0.3	
		Strawberries	7 days at 4 °C	0.0	Verhaelen et al. (2012)
			7 days at 10 °C	0.3	
		Turkey	10 days at 7 °C	0.0	Lamhoujeb et al. (2008)
	HAV	Bottled water	360 days at 4 °C	0.68	Biziagos et al. (1988)
		Carrot	4 days at 6 °C	Complete inactivation	Croci et al. (2002)
		Fennel	7 days at 6 °C	Complete inactivation	Croci et al. (2002)
		Green onions	29 days at 3 °C	1.17 (±0.10)	Sun et al. (2012)
			16 days at 10 °C	1.15 (±0.15)	
		Lettuce	9 days at 6 °C	2.46 (±0.17)	Croci et al. (2002)
		Spinach	28 days at 5.4 °C	1	Shieh et al. (2009)
		Marinated mussels (pH 3.75)	28 days at 4 °C	1.7	Hewitt and Greening (2004)

berry mimicking the contamination of produce by food handler disks. Approximately a 2 log reduction was observed on lettuce after 7 days at 4 °C while a 2.5 log reduction was observed on strawberries after 6 days at 4 °C. MNV, NoV GI and GII showed greater viral persistence on raspberries as compared to strawberries, especially at 21 °C (Verhaelen et al. 2012).

Porcine sapovirus (SaV) is a culturable enteropathogenic calicivirus and it has recently used as norovirus surrogate to examine virus attachment to lettuce. Recently, Wang et al. (2012) showed that SaV remained infectious on lettuce after 1 week of storage at 4 °C. For other enteric viruses, Kurdziel et al. 2001 estimated the D-values (number of days after which the initial virus numbers had declined by 90 %) for poliovirus in various vegetables. The resulting D-values were 11.6 days for lettuce, 14.2 days for white cabbage, and no decline for green onion and fresh raspberries for 2 weeks. The survival of poliovirus was investigated in commercial yogurt, reporting infectious viruses after 24 days of storage at 4 °C (Strazynski et al. 2002).

Rotavirus SA-11 survived on lettuce, radish, and carrots for 25–30 days at 4 °C (Badawy et al. 1985) and coronavirus remained infectious for at least 14 days on lettuce surfaces under household refrigeration conditions (Mullis et al. 2012).

In general, the above-mentioned studies indicated that enteric viruses will survive on chilled food over the periods before deterioration of the specified food.

4.2. Stability of Enteric Viruses Under Frozen Storage

The occurrence of enteric virus outbreaks caused by to the consumption of berries and shellfish that had been frozen several months (Bosch et al. 2001; Hutin et al. 1999; Niu et al. 1992; Pintó et al. 2009; Ramsay and Upton 1989; Reid and Robinson 1987; Sanchez et al. 2002) (Table 13.1) indicates that if food is contaminated before freezing, substantial fractions of the viruses will remain infectious during frozen storage. For instance, six NoV outbreaks occurred in Europe in 2005 and involved up to 1100 people and were associated with the consumption of frozen berries imported from Poland (Falkenhorst et al. 2005; Korsager et al. 2005). The occurrence of virus outbreaks linked to imported strawberries from China, has called the attention of The European Commission. The European Commission (SANCO 12655/2012) has implemented the monitoring of NoV and HAV in some food imports in accordance with Art 15(5) of Regulation (EC) No 882/2004 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:194:0011:0021:EN:PDF), employing the standardized CEN methodologies (ISO/TS 15216–1 and ISO/TS 15216–2; 2013).

Butot et al. (2008) extensively investigated the survival of HAV, NoV, RV and FCV, on frozen strawberries, blueberries, raspberries, parsley, and basil, concluding that frozen storage for 3 months had limited effects on HAV and RV infectivity in all tested food products, whereas in frozen raspberries and strawberries FCV infectivity showed the highest decay rate due to acid

pH. Persistence of NoV was evaluated by RT-qPCR, showing that NoV GII was less resistant than NoV GI under the tested conditions. However there was no more than 1 log difference in the reductions found for the two NoV genogroups. Likewise, after freezing of inoculated pizza product (7 and 14 days, at −18 °C) and mincemeat (8 days, −18 °C), no significant reductions in the NoV titer pretreated with RNase was observed (Mormann et al. 2010). Moreover, HAV was recently detected and typed from samples of mixed frozen berries linked to an Italian hepatitis A outbreak in 2013 (Chiapponi et al. 2014).

On shellfish, some studies have reported the presence of enteric viruses in frozen shellfish. For instance, Shieh et al. (2007) were able to detect and type HAV sequences in oysters implicated in an outbreak. These oysters were stored in the cold for 12 days and then frozen at −20 °C for 7 weeks before analysis. Sanchez et al. (2002) and Pintó et al. (2009) also detected and typed HAV from imported frozen clams that caused two outbreaks in Spain. All these results indicate that freezing has little or no effect on HAV infectivity in molluscan shellfish.

Overall, these studies showed that freezing does not ensure an adequate reduction of enteric virus if present in foods.

4.3. Effects of Relative Humidity on Enteric Virus Persistence

The influence of relative humidity on the survival of enteric viruses on different vegetables and fruits has scarcely been investigated. Stine et al. (2005) investigated the survival of HAV and FCV on lettuce, bell peppers and cantaloupe, stored at 22 °C under high (mean, 85.7–90.3 %) and low (mean, 45.1–48.4 %) relative humidity. HAV survived significantly longer than FCV, and high inactivation rates were reported under conditions of high humidity.

4.4. Stability of Enteric Viruses on Dried Food Products

Enteric virus survival in dried state has been studied mostly on inanimate surfaces or fomites, and has been reviewed earlier in this chapter. The multistate outbreak of hepatitis A associated with the consumption of sun-dried tomatoes shows that if food is contaminated before drying, substantial numbers of viruses will still remain infectious (Gallot et al. 2011; Petrignani et al. 2010).

4.5. Stability of Enteric Viruses Under Modified Atmosphere Packaging

Modified atmosphere packaging (MAP) is typically employed to slow the respiration rate of vegetables and fruits and therefore reduce the metabolism and maturation of the food products. Moreover, MAP is a way of extending the shelf life of fresh food products by inhibiting spoilage by bacteria and fungi. This technology replaces the atmospheric air inside a package with a protective gas mix. Overall this type of packaging is designed to inhibit bacterial or fungal growth and therefore is not effective against enteric viruses because they do not grow in food products. This is supported by recent reports

were the presence of enteric viruses were detected in ready-to-eat packaged leafy greens. In Canada, NoV were detected on 6 % and rotavirus in 0.4 % of lots tested from retail markets in southern Ontario. Packages with confirmed positive samples were imported into Canada (Mattison et al. 2010).

So far, only one study has evaluated the effect of various modified atmospheres on the survival of HAV on lettuce stored at room temperature and 4 °C for up to 12 days in ambient air and under various modified atmospheres (Bidawid et al. 2001a, b). The lettuce samples were stored in heat-sealed bags with the following percentages of gas mixtures (carbon dioxide [CO_2]:nitrogen): 30:70, 50:50, 70:30, and 100 % CO_2. Only at 70 % CO_2 at room temperature was a significant decline in virus survival observed. Because most commercially distributed vegetables are stored at lower CO_2 concentrations and at 4 °C, standard MAP conditions will not prevent HAV transmission.

4.6. Effects of Acidification on Enteric Virus Survival

Sauces, dressings, marinades, and similar food products depend on their acidity to prevent spoilage. They may consist of naturally acidic foods, such as tomatoes' sauce or fruit juices, or they may be formulated by combining acidic foods with other foods to achieve the desired acidity. Moreover some foods, such as vinegar and certain pickled vegetables, may develop acidity from microbial fermentation. However acidification of food is not a suitable hurdle to control enteric virus in foods since they are highly stable at an acidic pH. For instance, HAV had a high residual infectivity after 2 h of exposure to pH 1 at room temperature, remaining infectious for up to 5 h. HAV remained infectious for 90 min at pH 1 and 38 °C (Scholz et al. 1989). Similar trends are reported for human NoV, (Mormann et al. 2010) reported only a 1.7 log reduction of NoV titers pretreated with RNase after storage at 6 °C during 24 days under acid pH conditions in potato salad (pH from 5.0 to 5.5). Furthermore, no reduction in the virus titer was observed for storage in noodle salad (24 days, pH from 5.0 to 5.5) or tomato ketchup (58 days, pH 4.5). In conclusion these data show that acidification is not a suitable strategy to reduce the number of enteric viruses present on food.

5. CONCLUSIONS

Survival of enteric viruses in the environment and different food products has been well studied employing cell-adapted virus strains. However, there is a definite need for further research for the study of NoV survival. So far, NoV survival has been investigated either using surrogates or by molecular techniques. However both approaches have several drawbacks. Molecular techniques did not differentiate between infectious and non-infectious viruses while many differences have been reported between the inactivation of NoV and its surrogates, thus questioning the validity of these surrogates. Clinical

trials may be the best option, however studies with volunteers still are not accepted in many countries.

Furthermore it should be recognized, that most of the studies on virus persistence were performed under laboratory conditions and that data obtained from these studies may not truly represent their behavior under actual field conditions. For instance, several studies have reported virus intake by vegetables, although almost all data on virus persistence on vegetables have been obtained by surface inoculation of the target virus.

Overall, data provided in this review shows that enteric viruses are very stable in the environment and in food products. As a consequence, emphasis should be on prevention of contamination by implementing good hygienic, agricultural, and manufacturing practices. Strategies to reduce the risk of foodborne outbreaks of enteric viruses should focus on preventing foods from becoming contaminated. In developing countries, young children should be kept away from areas where fresh produce is grown and harvested. This measure is important for hepatitis A infection, since in developing countries this disease is usually acquired during early childhood as an asymptomatic or mild infection. Education of workers, with an emphasis on hygiene; providing facilities for maintaining cleanliness; and the use of treated water in production and processing will be major deterrents to contamination of food with enteric viruses. Shellfish harvesting areas should be monitored for NoV and HAV contamination. Moreover, because enteric viruses are easily transferred from utensils/fomites to food/persons and vice versa, efforts have to be taken to prevent cross-contamination in the different scenarios.

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CHAPTER 14

Using Microbicidal Chemicals to Interrupt the Spread of Foodborne Viruses

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1. INTRODUCTION

The human health impact of foodborne viral infections can be substantial (Painter et al. 2013), even though fewer types of viruses than bacteria can spread via foods. General difficulties in recovering and identifying viruses from foods and clinical specimens collected during foodborne outbreaks grossly underestimate the true role of viruses as foodborne pathogens (Bresee et al. 2002; Sair et al. 2002; Koopmans et al. 2002; Baert et al. 2009a; Glass et al. 2009; Bosch et al. 2011; Scallan et al. 2011; Verhoef et al. 2013) reinforcing the need for proper inactivation of viruses before the food is consumed. Hepatitis A virus (HAV) is well-known as a foodborne pathogen, but human noroviruses (HuNoV) have now emerged as the most frequent cause of foodborne outbreaks of acute non-bacterial gastroenteritis (Bidawid 2013); they account for over 50 % of all foodborne illnesses in the U.S. (Scallan et al. 2011). While rotaviruses are also known to spread via foods (Sattar et al. 2001), their outbreaks are rarely reported in peer-reviewed literature. The other foodborne pathogen of emerging concern is hepatitis E virus (Miyashita et al. 2012; Sattar and Tetro 2001); further work on the microbicidal activity against this virus awaits *in vitro* culture. This chapter focuses on the testing and application of microbicidal chemicals (disinfectants and hand hygiene agents) to inactivate viruses on inanimate and animate food contact surfaces as well as for the decontamination of foods consumed raw or with minimal processing. Table 14.1 defines the common terms used in this chapter.

2. BASIC CONSIDERATIONS

In spite of the wide-spread use of microbicides in reducing the risk from foodborne infections, several aspects of this practice are based on tradition rather than on a sound scientific basis (Sattar and Bidawid 2001; Sattar and Springthorpe 1996). In particular, the following must be clearly understood for any successful strategy to interrupt the spread of foodborne viruses using microbicidal chemicals (Bidawid 2013):

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Table 14.1 Glossary of common terms used in this chapter

Term	*Definition*
Antimicrobial agent	A physical or chemical agent that kills microorganisms or suppresses their growth
Antiseptic	An agent that destroys pathogenic or potentially pathogenic microorganisms on living skin or mucous membranes
Carrier	An inanimate surface or object inoculated with a test organism
Cleaning	Removing, by physical and/or chemical means, visible soil, dirt or organic debris from a surface or object
Microbial contamination	The presence of viable microorganisms in or on a given material or object
Decontamination	Freeing a person, object or surface of harmful microorganisms, chemicals or radioactive materials
Disinfectant	A physical or chemical agent that destroys pathogenic or potentially pathogenic microorganisms in or on inanimate surfaces or objects
EBSS	Earle's Balanced Salt Solution
Eluate	An eluent that contains microorganism(s) recovered from a carrier
Eluent	Any solution that is harmless to the test organism(s) and that is added to a carrier to recover the organism(s) in or on it
Chemical microbicide	A chemical that kills pathogenic or potentially pathogenic microorganisms in or on inanimate surfaces/objects and on living skin/mucous membranes
High-level disinfectant	A chemical or a mixture of chemicals that is bactericidal, fungicidal, and virucidal; may also be sporicidal with an extended contact time
Intermediate-level disinfectant	A chemical or a mixture of chemicals that is bactericidal, fungicidal, mycobactericidal and virucidal, but not sporicidal
Label	Written, printed, or graphic matter on, or attached to, the microbicide containers or wrappers
Low-level disinfectant	A chemical or a mixture of chemicals that kills only vegetative bacteria and enveloped viruses
Microbicide (microbiocide)	A physical or chemical agent that kills microorganisms
Neutralization	Quenching the antimicrobial activity of a test formulation by dilution of the organism/test formulation mixture and/or addition of one or more chemical neutralizers to the mixture
OTC	Over-the-counter topicals
Pathogen	Any disease-causing microorganism
Pesticide	Any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest
Potency	The degree of strength or power of a microbicide to render disease-causing microorganisms non-infectious
QCT-1	Quantitative carrier test—tier 1
QCT-2	Quantitative carrier test—tier 2
Sanitization	A process that reduces the microbial load on a surface or object

(continued)

Table 14.1 (continued)

Term	*Definition*
Soil load	A solution of one or more organic and/or inorganic substances added to the suspension of the test organism to simulate the presence of body secretions, excretions, or other extraneous substances
Sterile	Free from living microorganisms
Sterilization	A process that kills all forms of microbial life
Stringency of test method	The level of rigor, strictness or severity built into the method to reflect factors the test formulation may encounter under in-use conditions
Test formulation	A formulation that incorporates antimicrobial ingredients
$TCID_{50}$ (50 % tissue culture infective dose)	The dilution at which 50 % of all infected cell cultures show evidence of virus infection
Test organism	An organism that has readily identifiable characteristics. It also may be referred to as a surrogate or a marker organism
Use-dilution	The level to which a concentrated microbicide is diluted for use
Virucide (viricide)	An antimicrobial agent that kills (inactivates) viruses
Water hardness	The measure of the amount of metallic (e.g., calcium) salts in water

1. Important foodborne viruses, for example hepatitis A virus (HAV), can survive better than many enteric bacteria on inanimate and animate surfaces (Sattar et al. 2000b).
2. The in-use concentration of a given microbicide may be less effective against non-enveloped viruses in particular as compared to many foodborne vegetative bacteria (Ansari et al. 1989).
3. Unlike many types of bacteria, viruses cannot replicate in contaminated foods; thus, holding of foods at an inappropriate temperature as such is not a risk factor in case of viral contamination. But viruses may remain viable in contaminated foods for several days to months, especially under refrigeration.
4. Not unlike other types of pathogens, viruses in shellfish from fecally-polluted waters do not lend themselves readily to decontamination by microbicides.
5. Hands can readily acquire or donate infectious virus particles during handling and preparation of foods (Bidawid et al. 2004; Sharps et al. 2012; Stals et al. 2013).
6. Suitable microbicides, when properly used, can interrupt the transfer of viruses from contaminated surfaces to foods (Bentley et al. 2012; Fraisse et al. 2011; Girard et al. 2010; Kim et al. 2012; Mattison et al. 2007; Park et al. 2010).
7. Safety considerations exclude the use of certain types of microbicides (e.g., phenolics) on food contact surfaces (Gulati et al. 2001).

8. Microbicides often used on food contact surfaces are neither required to nor are tested against common types of foodborne viruses.
9. In the U.S., there are no officially-accepted methods for evaluating the virucidal activity of handwash and handrub agents; nor is there any regulatory framework to allow such products to make claims against viruses. Health Canada now has guidelines for testing such agents against bacteria, as well as viruses (Health Canada 2009).
10. Recognized flaws in current methods to assess the virucidal activity of microbicides to be used on environmental surfaces compromise the reliability of label claims of environmental surface disinfectants (Sattar 2006).

3. TEST METHODOLOGIES TO DETERMINE VIRUCIDAL ACTIVITY

The virucidal potential of microbicides is normally assessed by 'suspension' or 'carrier' tests (Maillard et al. 2013; Sattar and Springthorpe 1999). In suspension tests, a known volume of the challenge virus, with or without a soil load, is mixed with a 5- to 10-fold larger volume of the test microbicide (Springthorpe et al. 1986). For control, the virus is suspended in an equivalent volume of a liquid known to be harmless to the virus and its host cells. The mixtures are held for a defined contact time at a specified temperature, neutralized to stop virucidal activity, titrated for infectious virus, and the degree of loss in virus viability calculated. While suspension tests are easier to perform, they are also easier to pass (Abad et al. 1997; Sattar et al. 1986) and are thus suitable for screening the activity of microbicides under development. Regulatory agencies in North America do not accept claims of virucidal activity based on suspension tests for product registration purposes.

Under most field conditions the target virus is present on an animate or inanimate food contact surface. In view of this, carrier tests, where the challenge virus is first dried on a representative surface and then exposed to the test formulation, are considered more suitable in assessing the potential of microbicides under in-use conditions (Maillard et al. 2013).

4. FACTORS IN TESTING FOR VIRUCIDAL ACTIVITY

4.1. Test Viruses

The U.S. Environmental Protection Agency (EPA) requires ASTM International's carrier test method E1053 (ASTM International 2011b) to test environmental surface disinfectants (U.S. EPA 2012). Testing against each specific virus is required for listing on the label except in the case of hepatitis B virus (HBV), hepatitis C virus (HCV), and norovirus, for which the duck

HBV, bovine viral diarrhea virus, and the feline calicivirus, respectively, are currently considered acceptable surrogates for testing. This may change soon in view of the recent introduction of a guideline from the Organization for Economic Cooperation and Development (OECD 2013). While Health Canada also accepts the above-mentioned surrogates, for a general virucidal claim it requires a given product to show the required level of activity against the Sabin strain of poliovirus type 1 (Health Canada 2007). The use of this non-enveloped virus, which is also safe to handle and is relatively resistant to microbicides, makes product development easier and label claims simpler and reliable. However, one or more substitutes for poliovirus may be needed soon in view of the anticipated eradication of poliomyelitis and the expected ban on the laboratory use of all types of polioviruses (Mundel and Orenstein 2013).

What should one look for in selecting viruses to assess the activity of microbicides against foodborne viruses? Fortunately, the list of major foodborne viruses is short and identification of potential surrogates for them is relatively easy. The two most suitable viruses in this regard would be the cell culture-adapted strains of HAV (e.g., HM-175) and the F9 strain of feline calicivirus (FCV). Indeed, the feasibility of using such strains in testing microbicides used in settings where foods are processed and handled has already been demonstrated (Doultree et al. 1999; Gulati et al. 2001; Sattar et al. 2000a). The cell culture-adapted WA strain of human rotavirus (HRV) can also be used to evaluate microbicides against them (Sattar et al. 1994). The recent advent of cell culture-adapted strains of the murine norovirus (MNV) offers an alternative to testing microbicides against human norovirus (HuNoV; Sattar et al. 2011). Although FCV has been considered a suitable surrogate for human norovirus, its higher sensitivity to acidity than MNV may require care in testing acid-based formulations (Cannon et al. 2006; D'Souza et al. 2006).

The HAV shows the highest level of microbicide resistance of the foodborne viruses tested so far (Mbithi et al. 1992; 1993) and it would thus make a good surrogate if the selection were to be based on this factor alone. Working with this virus has become safer as effective vaccines against it are now available. The possible drawbacks in the use of HAV are that the turn-around time for test results is at least 1 week and that many formulations in current use may fail against this virus. This points to the need for further discussions on the justification of using one or more surrogates in testing microbicides against foodborne viruses and a consensus between major stake-holders is needed on which virus(es) may be the most suitable for this purpose (D'Souza and Su 2010; Park et al. 2010; Casteel et al. 2008).

4.2. Nature and Design of Carriers

The three categories of surfaces to be discussed here are: inanimate non-porous environmental items that may contact foods during storage, preparation and serving; fruits and vegetables that are consumed raw or with minimal processing; and hands of food handlers.

4.2.1. Environmental Surfaces

Food contact surfaces vary widely in their nature, usage, and level of cleanliness. The microtopography of a given surface may also change with the type and extent of use, thus providing either more or less protection to viruses deposited on it. Since it is impractical to test microbicides on all types of food contact surfaces prior to product registration, it would be logical to select and use a 'surrogate' surface. The selection of such a surrogate, inanimate, food-contact surface should consider the following: (a) how frequently it contacts foods and hands of food handlers, (b) how readily it releases infectious viruses it may carry, (c) it must not inactivate the test virus or irreversibly bind or sequester it such that virus elution from it becomes difficult, (d) its surface should be uneven enough to represent those in the field, (e) if meant for reuse, it should readily withstand repeated decontamination and sterilization, and (f) it should be resistant to microbicides commonly used in decontamination of food contact surfaces. Hierarchy of microbicide resistance of viruses has been proposed as an approach to assess the activity of environmental surface disinfectants against new and still fully uncharacterized viruses (Sattar 2007).

Further, any carriers made out of such a surrogate material should allow the convenient deposition of the desired volume of the test virus as well as the test microbicide and the entire carrier should be submersible in a reasonably small volume of the eluent without any wash-off. The need for keeping the eluent volume per carrier as small as possible is particularly relevant when working with viruses, because, unlike tests against bacteria, membrane filtration cannot be readily used to trap viruses from large volumes of eluates. The need for cell cultures for detection and quantitation of infectious virus in test samples also restricts the eluate volumes that can be easily and economically processed.

Disks (~1 cm in diameter and 0.7 mm thick) of brushed stainless steel offer all the desired attributes of a surrogate surface in testing microbicides against foodborne viruses (Sattar and Springthorpe 2001a, b). The microtopography of the disk surface is sufficiently uneven and the carriers can be handled in a closed system so that wash-off of the test virus does not occur. If needed, disks similar in size to those described above can be readily prepared from other types of food contact surfaces. Porous materials can also be made into disks as carriers (Traoré et al. 2002), but they are generally more difficult to work with in testing microbicides because their absorbent nature reduces the efficiency of recovery of test organisms. Besides, such materials are rarely meant to be decontaminated using microbicides. In addition to the above mentioned surfaces, investigators have recently also used different carrier surfaces, such as glass, vinyl, forks, knives, ceramic tiles, and PVC plastics in their inactivation and/or decontamination studies (Bentley et al. 2012).

4.2.2. Food Items

In view of the potential of fresh produce to spread viruses (Bidawid. 2013; Painter, et al. 2013; Seymour and Appleton 2001) such items may be treated with microbicides before consumption (Beuchat 2001). The use of

microbicides for this purpose requires the evaluation of their virucidal efficacy on representative types of vegetables and fruits which are eaten raw or after minimal processing. A carrier test using small disks or pieces of items such as lettuce or strawberries represents a feasible approach (Hirneisen et al. 2011; Bae et al. 2011; Fraisse et al. 2011; Casteel et al. 2009; Baert et al. 2009b; Butot et al. 2008; Mattison et al. 2007; Bidawid et al. 2000).

4.2.3. Hands

While virucides intended for use on human skin are often tested using hard inanimate surfaces, comparative testing has found skin to present a stronger challenge to microbicides (Woolwine and Gerberding 1995). This reinforces the need for using carriers of a suitable animate surface for evaluating the virucidal activity of formulations for the decontamination of hands. Virucides can be tested using the entire surface of both hands of an adult (ASTM 2013), but the disadvantages of such an approach include high variability in results, inability to run controls and test samples simultaneously, lack of statistical power, and the need for larger volumes of high-titered virus pools (Sattar and Ansari 2002). The fingerpad method (Ansari et al. 1989), which is a standard of ASTM International (ASTM 2010), avoids these drawbacks by using the thumb- and fingerpads of adults as *in vivo* carriers. In this method the test virus is placed on targeted areas on the hands, allowed to dry and then exposed to a handwash or handrub formulation for a suitable contact time. It also allows for the determination of reduction in virus infectivity after exposure to the test formulation alone or following post-treatment water rinsing and with or without drying using cloth, paper or warm-air (Ansari et al. 1991). The fingerpad method has already been applied to assess the microbicidal activity of foodborne viruses such as HAV (Mbithi et al. 1992), FCV (Bidawid et al. 2004), MNV (Liu et al. 2010; Sattar et al. 2011); and HRV (Ansari et al. 1988); others have used the whole hand approach (Liu et al. 2010; Lages et al. 2008).

4.3. Nature and Level of Soil Loading

The organic matrix or 'soil' surrounding viruses, whether they are in body fluids, food residues or sewage/sludge, enhances their survival in the environment. Normal precleaning of surfaces and items to be disinfected may reduce the amount of such soil but enough of it still remains and can interfere with the activity of the applied microbicide by either binding to it or by preventing its access to the target virus. Any good method for virucidal activity must, therefore, simulate the presence of such soil by incorporating in the test virus suspension a certain amount of organic and inorganic material, and this is now a requirement in several standard protocols (ASTM 2011c, 2013).

While many different types and levels of substances are used as the soil load in testing microbicides, extra precautions are needed in their selection and use when working with viruses. For example, animal sera may contain specific antibodies or non-specific inhibitors against viruses such as rotaviruses.

Fecal suspensions, which have been used in testing microbicides against HAV (Mbithi et al. 1990), are inherently variable and thus unsuitable as a soil load for standardized test protocols. To overcome these difficulties, a 'universal' soil load has been developed for testing microbicides against viruses as well as other pathogens (ASTM 2011c; Springthorpe and Sattar 2003; OECD 2013); it consists of a mixture of bovine mucin, tryptone (or yeast extract) and bovine albumin in phosphate buffer. The concentrations and ratios of the three ingredients are designed to provide a challenge roughly equivalent to that in 5–10 % bovine serum. This soil has been found to be compatible with all types of viruses, their respective host cells as well as other types of pathogens tested thus far (Sattar et al. 2003).

4.4. Diluent for Test Microbicide

Many microbicides are tested by manufacturers using distilled water as the product diluent, and since this is not clearly specified in label directions, most users use tap water instead. Formulations with marginal virucidal activity may work with distilled water, but fail when tap water is used as the diluent (Sattar et al. 1983). This highlights the importance of choosing the right diluent during product development and to clearly specifying it on the label.

Although tap water is commonly used in the field and may represent a stronger challenge to microbicides under test, it is unsuitable as a diluent in standardized tests for virucidal activity. This is because the quality of tap water as well as the nature and levels of disinfectants in tap water vary both temporally and geographically. In view of this, water with a standard hardness level of 200–400 parts per million $CaCO_3$ is considered a more desirable diluent in such tests (ASTM 2011c; OECD 2013; Sattar and Springthorpe 2001a).

4.5. Dried Virus Inoculum as the Challenge

As compared to a suspension test, a carrier with the test inoculum dried on it presents a stronger challenge to the microbicide being evaluated. While this may be possible with some viruses, certain commonly used surrogates (e.g., polioviruses) may lose ≥ 2 $\log_{10}$ of infectivity on drying (Mbithi et al. 1991), especially at low levels of relative humidity (RH). A fine balance may therefore be required to achieve the right degree of drying of the virus inoculum on carriers or by selecting a surrogate that is more stable during the drying of the test inocula. HAV, FCV, MNV and HRV are all more resistant to drying than enteroviruses in general (Sattar and Ansari 2002). Suitable controls must be included to determine the loss in the infectivity of the test virus during the drying process and the level that survives becomes the baseline for measuring the extent of virus inactivation by the test formulation (Springthorpe and Sattar 2003).

4.6. Time and Temperature for Virus-Microbicide Contact

Except for products which are meant for prolonged soaking of items for decontamination, the contact between the target virus(es) on an environmental surface and the microbicide under in-use conditions is generally very brief. This should be properly reflected in the design of a carrier test for virucidal activity and such contact times should not be longer than about 3 min to allow for relatively slow-acting but commonly used actives such as ethanol.

Formulations to be used on environmental surfaces are tested at an air temperature of 20 °C; this is lower than the ambient temperature indoors in many work settings and requires the use of suitable climate control chambers or water baths to maintain the desired temperature. Air temperatures higher than 20 °C may enhance the activity of microbicides while also accelerating the rate of their evaporation from the carrier surface. Products to be used outdoors during winter months or indoors in refrigerators must be shown to be effective against viruses at lower temperatures.

4.7. Elimination of Cytotoxicity

Cytotoxicity of the test formulation to host cells is an important factor in tests for virucidal activity because it can interfere with the reading and interpretation of test results. In addition, any material and procedure used to remove and/or neutralize cytotoxicity must themselves be safe for the test virus and its host.

A 10- to 100-fold dilution of the virus-microbicide mixture at the end of the contact time is one simple and potentially viable approach to reducing cytotoxicity (Lloyd-Evans et al. 1986). This approach, however, requires relatively high titered pools of the test virus and may not work on its own for chemicals which are highly cytotoxic. Microbicides such as formaldehyde can effectively kill host cells without detaching them or producing any apparent damage to them. Such cytotoxicity can be misleading because host cell monolayers may appear undamaged but are unable to support virus replication. Moreover, one should note that even when toxicity appears to be visibly removed, subtle effects on the cells and potentially on their ability to support virus replication, may remain. This needs to be examined through a low-level virus challenge (Sattar et al. 2003).

Gel filtration (ASTM 2012) or ultracentrifugation (Doultree et al. 1999) of virus-microbicide mixtures may be effective in the removal of cytotoxicity but such steps invariably extend the contact of the virus with the test microbicide by several minutes or more and bring into question the validity of label claims of virucidal activity for many applications. Other considerations in the selection and use of procedures for the elimination of cytotoxicity have been described before (Sattar and Springthorpe 2001a).

4.8. Neutralization of Virucidal Activity

For accurate and reproducible results, the microbicidal activity of the test formulation must be arrested immediately at the end of the contact time (Sutton 1996). This can be achieved by either the addition of a neutralizer

or dilution of the virus-microbicide mixture or a combination of both. Whichever approach is adopted, its effectiveness must be properly validated before the test results can be considered valid.

The difficulties in choosing a suitable chemical neutralizer are somewhat similar to those enumerated above for cytotoxicity removal. While a 100-fold dilution of the virus-microbicide mixture soon after the end of the contact time has proven effective in dealing with most types of microbicides (Lloyd-Evans et al. 1986), this procedure requires that the volume of the diluent be kept relatively small to allow for the titration of most of the eluate.

4.9. Quantitation of Virus Infectivity

The availability of a simple and reproducible method for assaying infectious virus in the test and control samples is absolutely essential for determining the level of virucidal activity. Indirect measures of virus infectivity based on assays for antigens, enzymes or nucleic acids are not recommended because of the lack of demonstrated correspondence between their concentrations and those of infectious virus in the samples being assayed (Fraisse et al. 2011).

It is noteworthy that the presence of microbicide residues, even in diluted eluates, may increase or decrease the susceptibility of the host cells to the test virus. In case of decreased susceptibility, the host system could overestimate the activity of the tested microbicides by not being able to detect the presence of low levels of infectious virus in the inoculum. An increase in the level of infectivity could possibly be due to any one or a combination of: (a) unmasking of more viral receptors on the host cell surface, (b) inactivation of specific or non-specific virus inhibitors, (c) altering the electrostatic charges on the virus and/or the cell surface, and (d) deaggregation of viral clumps. Controls must, therefore, be included in virucidal tests to rule out the presence of such interference and for the results to be considered valid. The best way to approach this is to first expose the cell monolayer to a non-cytotoxic level of the test microbicide and subsequently challenge the cells to the test virus diluted to yield countable infectious foci such as plaques. If the number of infectious foci in such pre-exposed monolayers is not statistically different from that in the monolayers treated with a control fluid, the product can be assumed to be free from such interference.

4.10. Number of Test and Control Carriers

High enough numbers of test and control carriers must be included to make the results statistically meaningful. This requires some knowledge of the degree of reproducibility of the assay methods; since viruses require a host system, the results tend to be inherently more variable than those observed for bacteria and fungi. In general, methods which determine virus plaque- or focus-forming units are more accurate than the most probable number (MPN) techniques. Each measure of reduction in virus infectivity by a microbicide is obtained by comparison with controls not exposed to microbicide. Therefore, it is

important that sufficient numbers of such controls are included to obtain an accurate mean value against which each test carrier can be assessed.

4.11. Product Performance Criteria

For government registration, microbicidal products must meet a performance criterion which is based on practical considerations rather than on sound public health science. A 3–4 $\log_{10}$ reduction in virus infectivity titer after exposure to the test formulation is regarded as satisfactory virucidal activity. This criterion is lower than the minimum 5–6 $\log_{10}$ reductions required for other classes of pathogens because of the general difficulties in generating high-titered virus pools. The OECD guideline (2013) requires a $\geq$4 $\log_{10}$ reduction in virus infectivity.

5. CURRENTLY AVAILABLE TESTS

Table 14.2 lists the methods currently accepted as standard for testing the virucidal activity of microbicides for use on environmental surfaces and human hands.

5.1. Quantitative Suspension Tests

The ASTM suspension test (ASTM 2011a) for virucidal activity (E1052) is for special applications of virucides such as inactivation of viruses in contaminated wastes and as a first step in determining the virucidal potential of liquid chemical microbicides, liquid hand soaps, over-the-counter (OTC) topical products or other skin care products.

Another quantitative suspension test for virucidal activity of chemical disinfectants and antiseptics is being drafted by Comité Européen de Normalization's Technical Committee (CEN). An adenovirus and a vaccine strain of poliovirus are listed as test viruses. The contact time at 20 ± 1 °C ranges from 30 to 60 min depending on the intended use of the product. The formula being evaluated is tested with and without an added protein load in the form of either 0.3 % bovine serum albumin or 5 % defibrinated sheep blood. The product performance criterion is a minimum 4 $\log_{10}$ reduction in the infectivity titer of the test virus.

5.2. Quantitative Carrier Tests

There are four methods in this category in North America (Table 14.2). The first is an ASTM (#E1053) standard (ASTM 2011b), which has been revised recently. It is meant for evaluating the activity of liquid or pressurized antimicrobials against viruses on non-porous, inanimate environmental surfaces. This standard lists ten different viruses with varying degrees of resistance to liquid chemical microbicides. It recommends, however, that the test formulation be evaluated at least against a poliovirus, a herpesvirus and an

Table 14.2 Standard test methods for evaluating the virucidal activity of microbicides designed to be used on environmental surfaces or human hands

Organization	*Title of standard*	*Document #*	*Reference #*
ASTM International	Standard test method for determining the virus-eliminating effectiveness of hygienic handwash and handrub agents using the fingerpads of adults	E1838	ASTM (2010)
	Standard test method to assess the activity of microbicides against viruses in suspension	E1052	ASTM (2011a)
	Standard test method to assess virucidal activity of chemicals intended for disinfection of inanimate, nonporous environmental surfaces	E1053	ASTM (2011b)
	Standard quantitative disk carrier test method for determining the bactericidal, virucidal, fungicidal, mycobactericidal and sporicidal activities of liquid chemical germicides	E2197	ASTM (2011c)
	Standard practice for use of gel filtration columns for cytotoxicity reduction and neutralization	E1482	ASTM (2012)
	Standard test method for evaluation of hygienic handwash and handrub formulations for virus-eliminating activity using the entire hand	E2011	ASTM (2013)
Org. for Economic Co-op. & Develop. (OECD)	Guidance document on quantitative methods for evaluating the activity of microbicides used on hard non-porous surfaces; series on testing and assessment	No. 187, JT03342231; ENV/JM/MONO(2013)11	Org. for Economic Co-op. & Develop. (OECD) (2013)

adenovirus to qualify for a general virucidal claim. The test virus suspension is first dried on a glass Petri plate and then overlaid with a known volume of the test formulation for a predetermined contact time at ambient temperature. At the end of the contact time, a diluent is added to the virus-product mixture and the test surface is scraped to resuspend the virus film. The eluates and controls are assayed for infectious virus to determine the loss in virus titer

due to the test formulation's virucidal activity. Calf serum is recommended as soil (except for rotaviruses) and water with a specific level of hardness is to be used if the product requires dilution in water prior to use.

The ASTM E2197 (ASTM 2011c) is referenced as a test method for testing the virucidal activity of environmental surface disinfectants (Health Canada 2007). The methods, also referred to as the second tier of a quantitative carrier test (QCT-2) can be applied to all major classes of microorganisms including viruses (Springthorpe and Sattar 2005). This allows for direct comparison of results among different classes of microorganisms. The test uses flat stainless steel disk carriers (approx. 1 cm diameter) and a microbial inoculum of 10 µL. After drying of the inoculum the contaminated carrier is exposed to 50 µL of the test microbicide at 20 °C for the manufacturer's recommended contact time. The reaction is terminated by neutralization of the microbicide. In most cases neutralization is achieved by simple dilution with a physiological saline, but in some instances a chemical neutralizer is required prior to dilution. The test virus can then be titrated by standard methods. This method (E2197), now a standard of ASTM International (ASTM 2011c), has been used to test for virucidal activity of microbicidal chemicals against several types of viruses (Sattar et al. 2003; Maillard et al. 2013). In June 2013, OECD published a guide which includes a carrier test method for assessing environmental surface disinfectants against viruses (OECD 2013).

The third carrier test is the *in vivo* fingerpad method to assess the virus-eliminating activity of topicals. Full details of this method (#E1838) are given in the literature already cited above or in the ASTM standard itself (ASTM 2010). The fourth method is a standard (#E2011) for virucidal evaluation of formulations when these are performed on the whole hand (ASTM 2013).

6. PRACTICAL ASPECTS OF TESTING MICROBICIDES

We describe in this section the key practical considerations in evaluating microbicides against foodborne viruses. The methods described here are standards of ASTM International and have been used extensively in working with a variety of viruses (Springthorpe and Sattar 2003; Sattar et al. 2003; Ansari and Sattar 2002). Fig. 14.1 summarizes the basic steps in the quantitative carrier test method (ASTM 2011c) while Fig. 14.2 presents the basic steps in the fingerpad method (ASTM 2010). While no nationally- or internationally-accepted methods are available to assess the activity of microbicides used in the decontamination of fruit and vegetables, published studies (Bae et al. 2011; Baert et al. 2009b; Fraisse et al. 2011; Hirneisen et al. 2011; Casteel et al. 2008, 2009; Bidawid et al. 2000, 2004) should serve as a guide in the design and performance of such testing.

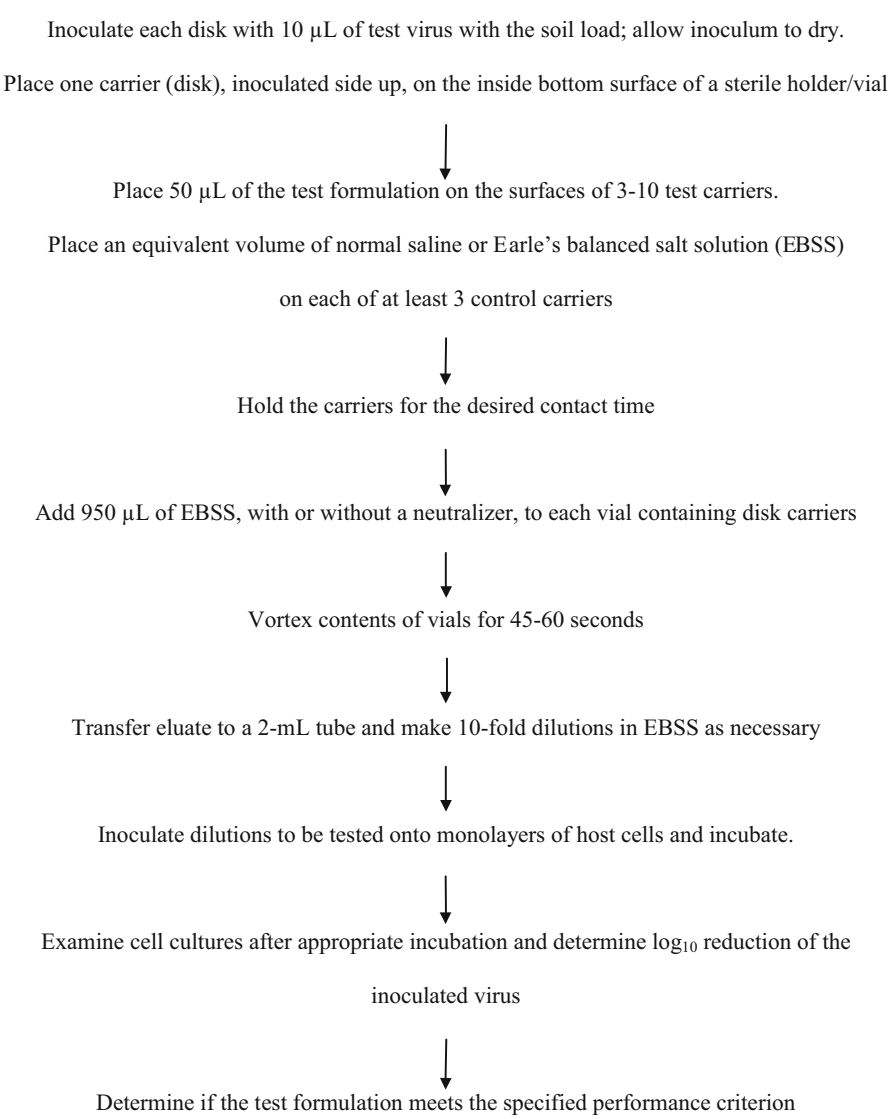

Figure 14.1 Basic steps in disk-based quantitative carrier test for virucidal activity (ASTM E-2197).

The three viruses described below have been selected based on their: (a) relevance as foodborne pathogens, (b) relative resistance to microbicides, (c) ability to withstand drying on environmental surfaces and human skin, (d) availability of cell culture-based infectivity assays, and (e) safety for work in experimental settings and for placement on the intact skin of consenting adult subjects.

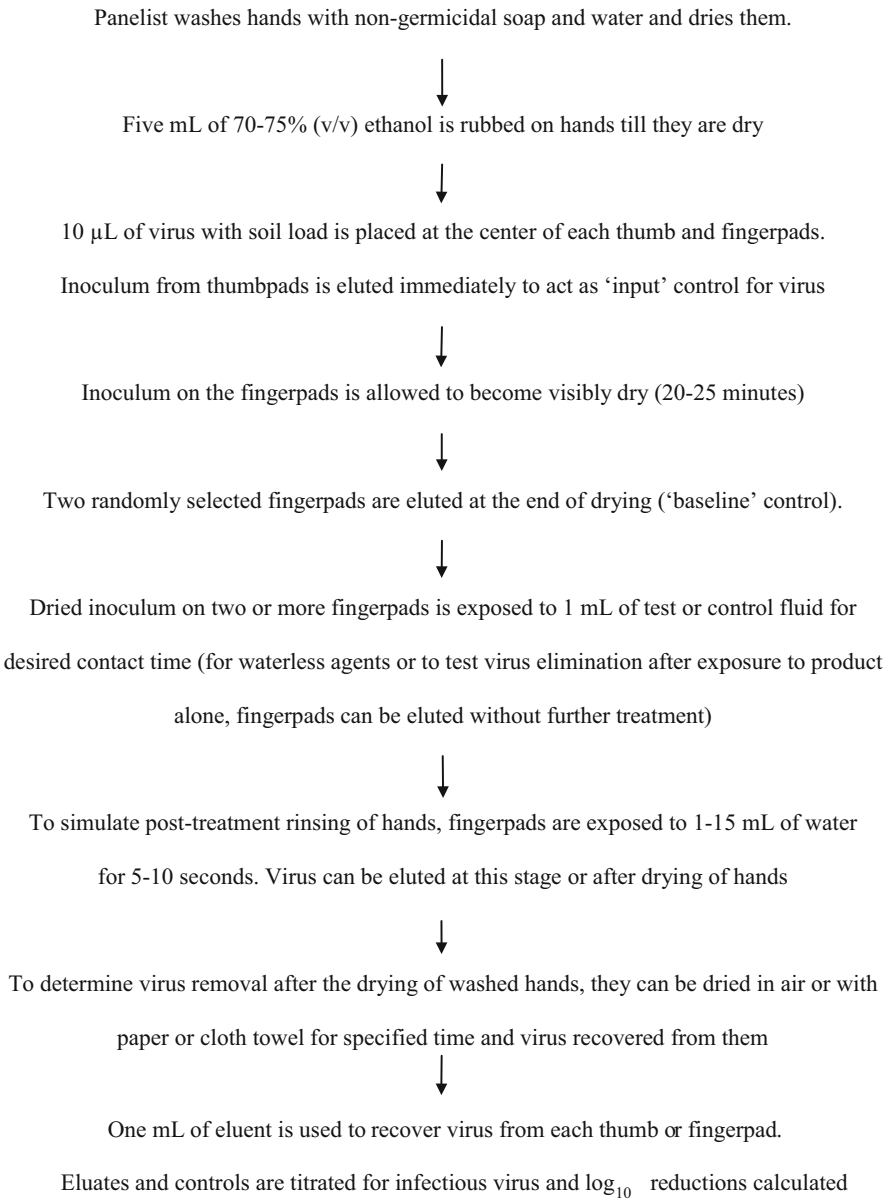

Figure 14.2 Basic steps in the fingerpad method (ASTM E-1838) for testing hand-wash or handrub agents against viruses.

The need for cell cultures adds an extra layer of difficulty when working with viruses. Also, procedures that work perfectly in one laboratory may not work elsewhere due to even slight variations in the quality of media/reagents

and in procedures for the clean-up and sterilization of labware, etc. Each laboratory must develop and document its own standard operating procedures for each host cell type and test virus to be used. However, regardless of the methods used for cell culture, preparation of virus pools, and quantitation of virus infectivity, the procedures for testing the activity of microbicides must adhere to the basic requirements as described above to ensure a sufficient level of stringency and reproducibility, and to allow the comparison of the results from tests using different viruses and test formulations.

While ultracentrifugation may be needed to increase the virus titer, the use of highly purified virus pools is not recommended for testing microbicides because such purification may enhance susceptibility of the virions to microbicides. Described below are some viruses that can be used in such tests.

6.1. Strain HM-175 (ATCC VR-1402) of HAV

HAV, an important foodborne pathogen, affects the liver and is excreted in the feces of infected individuals. It is relatively resistant to drying and mechanical damage and is also generally more resistant to microbicides than other non-enveloped viruses of human origin. Immunization of lab workers with the now available vaccines makes the handling of this virus much safer. The recommended cell line for making HAV pools and for performing infectivity titrations is FRhK-4 (ATCC CRL-1688). Six to seven days are needed to complete an infectivity assay due to the relatively slow rate of growth of the virus.

6.2. Strain F9 (ATCC VR-782) of FCV

FCV, pathogenic to cats but believed harmless to humans, belongs to a group of small, round viruses. FCV, which is non-enveloped, is related to HuNoV, a major cause of acute gastroenteritis in humans and also a significant foodborne pathogen. Since HuNoV cannot be grown *in vitro*, FCV is generally accepted as its surrogate and has been used in testing microbicides in settings where foods may be handled (Bidawid et al. 2004; Gulati et al. 2001). The cell line recommended for work with FCV is CrFK (ATCC CCL-94) and a plaque assay system based on these cells has been reported (Bidawid et al. 2003). This virus grows to high titers (~10^8 infective units/mL) within 28–36 h and produces visible CPE or plaques in less than 36 h. This is helpful for a rapid turn-around of results based on infectivity assays.

6.3. Murine Norovirus Type 1 (Strain S99)

MNV is an enteric virus that infects mice, and is transmitted through the fecal-oral route. It is most closely related to human noroviruses, both genetically and biochemically, and it is culturable in RAW 264.7 cells (Wobus et al. 2004; Sattar et al. 2011). Unlike FCV, however, MNV is more susceptible to alcohols and to

drying, but more resistant than FCV to low pH (Escudero et al. 2012; Seo et al. 2012; Park et al. 2010; Cannon et al. 2006; D'Souza et al. 2006). Therefore, MNV is now considered a more suitable surrogate for HuNoV due to its greater acid tolerance (Cannon et al. 2006). As of now, ATCC does list MNV. However, strains of the virus are available for experimental use from Washington University School of Medicine, St. Louis, Missouri, and the Friedrich-Loeffler-Institut, Greifswald, Germany.

6.4. Human Rotavirus WA Strain (ATCC VR-2018)

The Wa strain (ATCC VR-2018): Rotaviruses, which are a common cause of acute gastroenteritis in humans, are excreted in diarrheic feces in large numbers (Ward et al. 1991). Foodborne spread of rotaviruses has been documented (Sattar and Springthorpe 2001a). Recommended cell lines are MA-104 (CRL-2378) and CV-1 (ATCC CCL-70; Sattar et al. 2000a). Rotaviruses are safe for normal healthy adults as most adults have acquired immunity against them. The ability of rotaviruses to withstand drying also adds to their attraction as surrogates in testing microbicides.

Two important factors to note when working with rotaviruses are that: (a) many of them are inhibited by fetal bovine sera often used in cell culture, and (b) the presence of proteolytic enzymes such as trypsin is needed to promote rotavirus infection of host cells (Ramia and Sattar 1980).

6.5. Additional Controls in Virucidal Tests

The use of cell cultures requires the incorporation of the following additional controls in tests for virucidal activity (Sattar et al. 2002, 2003) because either the test substance or the neutralizer or both could alter the susceptibility of host cells to the virus in the test. These controls must be run initially at least once and may not need to be included in subsequent tests as long as the same cell line, virus, test formulation, neutralizer and method are in use.

Cytotoxicity Control: This control (a) determines the dilution of the test substance that causes no apparent degeneration (cytotoxicity) of the cell line to be used for measuring virus infectivity, and (b) assesses whether the neutralizer reduces or enhances such cytoxicity. For this control, make a 1:20 dilution, then a 1:200 dilution of the test substance in EBSS with and without the neutralizer. Remove the culture medium from the host cell monolayers and put into each the same volume of inoculum as used in virus assay; control monolayers receive an equivalent volume of EBSS only (without any neutralizer). Use at least three monolayers for controls as well as for each dilution of the test substance being assessed. Hold the cultures at room temperature for the same length of time as used for virus adsorption, then examine under an inverted microscope for any visible cytotoxicity.

If cytotoxicity is observed, a different neutralizer or alternative approaches to the removal/reduction of cytotoxicity may be needed. It is sometimes advisable to use gel filtration to remove the disinfectant (ASTM E1482), although this procedure may lengthen the exposure time of the test organism to the disinfectant. If no cytotoxicity is observed at either dilution, the test substance and the neutralizers should be subjected to the following interference test.

Control for Interference with Virus Infectivity: Levels of the test substance which show no obvious cytotoxicity could still reduce or enhance the ability of the challenge virus to infect or replicate in host cells, thus interfering with the estimation of its virucidal activity (Sattar et al. 2003). An interference control must, therefore, be included to rule out such a possibility. For this purpose, remove the culture medium from monolayers of the host cell line(s) and add a 1:20 dilution, or a dilution greater than the one that demonstrated cytotoxicity, of the test substance in EBSS to each of the test monolayers with and without neutralizer, using the same volume as that of the inoculum used in virus titration. Controls receive EBSS alone (without the neutralizer). Hold the monolayers at room temperature for the same length of time as used for virus adsorption and inoculate each with a low number (approx.10–20) of infective units of the challenge virus. Incubate the monolayers for virus adsorption, place maintenance medium in the cultures, incubate them for the time required for virus replication and then examine for cytopathology or foci of virus infection.

Any significant difference in virus infectivity titer is indicative of the ability of the test material or the neutralizer to affect the virus susceptibility of the host cells. In such case, a different neutralizer or alternative approaches to the removal of the residues of the test product may be needed. Both the cytotoxicity and interference controls must be included even when virus infectivity is titrated using the $TCID_{50}$ method.

Control Carriers: The minimum number of control carriers to be used in each test is three regardless of the number of test carriers. For control carriers, add 50 µL of EBSS instead of the test formulation. The contact time and temperature for the control carriers must be the same as those for the test carriers.

7. MICROBICIDES IN ENVIRONMENTAL CONTROL OF FOODBORNE VIRUSES

Table 14.3 summarizes the data from selected studies on the activity of microbicidal chemicals against a variety of foodborne viruses on different types of environmental surfaces, human hands and foods.

Table 14.3 Efficacy of various chemical disinfectants against various foodborne viruses on different types of surfaces, hands and foods

Chemical	*Concentration*	*Test virus*	*Contact time s = seconds; m = minutes*	*Surface/food*	*Log_{10} reduction*	*References*
Hand sanitizer (Ethanol)	62 %	MNV/FCV	30 s	Fingerpad	≤0.67	Liu et al. (2010), Lages et al. (2008)
	70 %	HuNoV/FCV/MNV	1–5 m	Fingerpad	2.67–3.6	Park et al. (2010)
	90 %	HuNoV/FCV/MNV	30 s	Suspension	<0.0/<0.5/2.0	Fraisse et al. (2011)
Soap + triclosan	0.10 %	FCV/MNV	1–5 m	Fingerpad	<1.3/>3.4	Liu et al. (2010), Lages et al. (2008)
Soap + triclosan	0.50 %	HuNoV	30 s	Fingerpad	<1.2	Park et al. (2010)
Peroxyacetic acid	250 mg/L	MNV	5 m	Lettuce	<2.00	Baert et al. (2009b)
Quaternary ammoniun compound (QAC)	220 ppm	MNV	30 s — 1 m	Ware-wash	<3.2	Feliciano et al. (2012)
QAC	4240 ppm 8480 ppm	HAV/FCV/MS2	10 m	Glass	0.34/3.3/1.26	Tung et al. (2013)
Chlorine dioxide	10 ppm 0.247 mg/L	FCV/HAV/RV MNV/MS2	10 m 1 m	Berries, herbs suspension	<2.0 4.0	Butot et al. (2008), Lim et al. (2010)
Trisodium phosphate	5 %	FCV/MS2/MNV	30 s–1 m	Formica coupons	>6.0 (FCV/MS2)/1.0 (MNV)	D'Souza and Su (2010)
Wash Detergent: – UltraKleen/Monsoon – Water + SAFE – Water	3000/100 ppm 0.1 % SAFE	MNV HuNoV	30–76 s 30 s 2 m 2 m	– Ware-wash – Finger Pad – Lettuce – Lettuce	<3.3 <1.58 <1.29 <1.15	Feliciano et al. (2012), Liu et al. (2010)
Na hypochlorite	200 ppm	HuNoV/MNV/FCV HAV/MS2	2–5 m	Berries, herbs, lettuce	<2.6	Baert et al. (2009b), Butot et al. (2008), Casteel et al. (2008)
Na hypochlorite	160–1600	HuNoV	5 m	fingerpad	5	Liu et al. (2010)

Table 14.3 (continued)

Chemical	*Concentration*	*Test virus*	*Contact time s = seconds; m = minutes*	*Surface/food*	*Log_{10} reduction*	*References*
Na hypochlorite	200 ppm 5000 ppm	FCV/MNV FCV/MNV	1–5 m	Steel coupons, ware-wash	1–3.5 5.2	Kim et al. (2012), Feliciano et al. (2012)
Na hypochlorite	20 ppm	HAV/MS2	5–10 m	Strawberries, tomato, lettuce	2	Casteel et al. (2008)
Washing with Bubbles – Bubbles + Chlorine – Bubbles + PPA	+ 15 ppm + 100 ppm	FCV/MNV/HAV	2 m	Lettuce	<1.00 2.9/1.4/1.9 3.4/2.3/0.7	Fraisse et al. (2011)
Hydrogen peroxide	30 % (w/w)	FCV	10–20 m	Stainless steel, glass, vinyl Floor, ceramic PVC plastic	4	Bentley et al. (2012)
Virkon®	0.25 % 0.5 % 1 %	ΦX-174/MS2/ FCV/HAV	10 m	Glass coverslips	>5.1/3.2/2.97/0.29 >5.1/4.4/2.98/0.56 >5.1/>5.19/ >4.22/>3.18	Solomon et al. (2009)

8. CONCLUDING REMARKS

Viruses continue to be important pathogens in general and as foodborne pathogens in particular, but our understanding of the actual sources of viral contamination in many foodborne outbreaks remains incomplete making it difficult to design and apply proper strategies to prevent and control the spread of such pathogens. However, hands are universally recognized as vehicles for the spread of a number of viruses. Successful strategies to prevent virus spread via these vehicles involve a sound hand decontamination protocol, diligently applied with a good topical agent. A lack of compliance with hand antisepsis guidelines and, perhaps the use of ineffective microbicides, continues to undermine the full potential of infection control and prevention measures in this regard. The ease with which washed hands can pick up infectious viruses upon contact with contaminated environmental surfaces and objects suggests that the emphasis on hand antisepsis should be combined with an awareness of the need for proper/regular cleaning and decontamination of those surfaces and objects that come in frequent contact with decontaminated hands.

Standardization of virucide tests, nationally and internationally, will promote confidence among microbicide users and the general public. This chapter provides the basis for a general understanding of the pitfalls in testing microbicides for their virucidal potential, and suggests the basic protocols and controls which should be present in generic methods. This should allow the reader to better understand this field and to be able to critique the published literature independently.

Standard tests for virucides are now available. These tests provide improved carrier design, better methods for cytotoxicity removal, a 'universal' soil load and other improvements. However, regulatory agencies, especially in the U.S., must soon decide on accepting surrogates in tests for virucidal activity and label claims and also set product performance standards. Health Canada (2007, 2009) and OECD (2013) already have these in place. Any such discussion must consider activity against one or more carefully selected non-enveloped viruses, representative of foodborne viral pathogens. Many products currently on the market list only enveloped viruses among the organisms on the label. Persons unfamiliar with virus classification can be easily misled by this, especially if the enveloped viruses listed are among those most feared.

Our current knowledge does not allow, with any degree of certainly, the determination of the desired level of reduction in virus load in a given setting to significantly reduce disease transmission. There are also obvious practical limitations to how high a level of challenge virus(es) one can present to the product under evaluation. By the same token, what would one regard as too low a level of challenge? Experience over the past three decades clearly indicates that if test viruses are chosen carefully, it is feasible to determine a 3–4 $\log_{10}$ reduction in virus infectivity titer after its exposure to a test microbicide in a proper carrier test. The viruses selected for QCT-2 are based on their (a)

relative safety for the laboratory staff, (b) ability to grow to titers sufficiently high for testing, (c) property to produce cytopathic effects or plaques, or both, in cell cultures, (d) potential to spread through contaminated environmental surfaces and medical devices, and (e) relatively high resistance to a variety of chemicals.

With these considerations and given the fact that enveloped viruses in general do not survive well on environmental surfaces and are more susceptible to microbicidal chemicals, all viruses included here are non-enveloped. Other strains or types of non-enveloped viruses may be substituted in the test provided they meet the preceding criteria.

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CHAPTER 15

Virus Inactivation During Food Processing

Alvin Lee and Stephen Grove

1. INTRODUCTION

Consumers are increasingly demanding food products that are fresh-tasting, nutritious and convenient. At the same time, consumer concerns about food safety have steadily increased as the incidence of reported foodborne illnesses has continued to rise. These trends have fueled interest in nonthermal processing technologies, such as high hydrostatic pressure processing, irradiation, pulsed electric fields, and high-intensity pulsed light. A nationwide consumer survey assessing awareness of alternative food processing technologies, consumer food safety attitudes and knowledge about nonthermal processed products, such as high pressure treated foods, indicated that a large proportion of polled consumers were willing to pay $0.25–0.50 more regardless of the value of the product if they were communicated on the benefits to the consumer (Hicks et al. 2009). Similarly, these trends have also fueled the renewed interest in wash water technologies such as more effective sanitizers that are natural and organic along with processing technologies that aid in the removal of contaminants to ensure the safety of fresh fruits and vegetables.

Although traditional thermal processing technologies or the application of heat to a food matrix are effective in inactivating most foodborne pathogens including viruses, it is often harsh and causes detrimental changes to foods. New preservation technologies that do not involve the use of heat are attractive because, other than causing limited detrimental effects on food quality, they can potentially be used to minimize or eliminate extensive thermal processing and the use of chemical preservatives. Preservation of freshness and protection of flavor, appearance and nutritional value results in a high quality food product, often with extended shelf-life. For these reasons, nonthermal processing technologies offer the ability to produce foods with improved quality, increased consumer appeal, and a value-added premium price. Although commercialization of these technologies has been slow to date, the above trends plus improvements in efficiency and reductions in cost mean that the rate of adoption of nonthermal processes is likely to increase.

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2. NONTHERMAL PRESERVATION PROCESSES

The use of novel processing technologies without the use of thermal energy to either improve or enhance the inactivation of microbial pathogens without compromising the visual and nutritive qualities of the food matrix has gained traction over recent years. The application of such technologies can cause changes to proteins and other food components, such as the denaturation of proteins by high pressure processing, resulting in texture changes and development of new products while maintaining fresh attributes of the product. A number of technologies that could be applied to various food types are available commercially, but in the scope of this chapter, non-thermal technologies will be highlighted. The list is by no means exhaustive.

It is difficult to educate a population on the health benefits of changing their eating habits from traditionally consuming raw shellfish to thoroughly cooking shellfish before consumption (Halliday et al. 1991; Salamina and D'Argenio 1998). An epidemiological survey conducted in Naples, Italy concluded that the common consumption of shellfish by the population was not affected by the awareness of the high incidence of hepatitis A infection in the region and knowledge of its route of transmission and that cooking shellfish prior to consumption was frequently insufficient to inactivate viruses (Salamina and D'Argenio 1998). Thus an alternative nonthermal or low-heat preservative process would be valuable to reliably improve the margin of safety associated with consumption of raw product. Due to the traditional consumption of oysters raw or minimally cooked, this process must not only ensure a microbiologically-safe food, but also provide a product that is almost identical to the raw product in terms of organoleptic quality. To ensure that water quality and good manufacturing practices are not compromised, the introduction of such a process must add to, and not replace, current standard procedures. The application of heat has long been recognized as a process that prolongs the shelf-life of foods while improving food safety, but in some products heating can cause undesirable changes affecting product organoleptic and nutritional qualities. Food textures are usually altered in some manner and some vitamins are known to degrade during thermal processing. Vegetable tissues are often softened by heat and application of chemicals may be required to regain firmness (San Martin et al. 2002).

2.1. High Pressure Processing

Today, high pressure processing (HPP) has become a commercial reality with several fruit- and vegetable-based refrigerated food products currently on the international market, including a range of juices and fruit smoothies, jams, applesauce-fruit blends, guacamole and other avocado products, tomato-based salsas and fajita meal kits containing acidified sliced capsicum and onions, and heat-and-serve beef or chicken slices (precooked). Additionally, ready-to-eat meat products and seafood, including oysters, are on the market

in the United States and Europe (Smelt 1998; Stewart and Cole 2001). A batch system is typically used where the product is placed into a final flexible consumer package before pressurization. The packages are loaded into a basket and placed into the pressure vessel, where they are submerged in a liquid of low compressibility (typically potable water). Once loaded and closed, pressures ranging from 100 to 700 MPa are normally generated by pumping additional water into the vessel. The process is relatively energy-efficient, requiring approximately the same energy to raise the pressure to 400 MPa as required to heat to 30 °C (Cheftel 1995). Once the desired target pressure is obtained, no further energy is required to sustain that pressure (Farr 1990). Unlike thermal processing, pressure is distributed instantaneously and uniformly throughout foods, ensuring a homogenous treatment regardless of the size or shape of the product (Hoover et al. 1989).

The treatment of foods with HPP is based on compressing the water surrounding the food (Barbosa-Canovas et al. 1998). Although its compressibility is low, the volume of water is decreased by 15 % at 600 MPa and 22 °C (Farr 1990). The compression of water causes a moderate increase in temperature (commonly referred to as adiabatic heat or the heat of compression), the extent of which is dependent on the initial temperature of the vessel and the rate of compression. When HPP is conducted at ambient or lower temperatures, there is no substantial rise in the temperature of the treated food. Decompression of the vessel reverses this effect at an equivalent rate (Cheftel 1995).

The primary advantages of HPP over thermal processing are the minimal chemical and physical effects exerted on most foods while imparting a microbial kill step. High pressure does cause a range of effects on the molecular interactions in foods. Ionic bonds and at least a proportion of hydrophobic interactions are broken or distorted by high pressure, whereas hydrogen bonds are strengthened (Hoover et al. 1989) and covalent bonds are unaffected (Ledward 1995). As a result of the pressure-induced changes to ionic bonds and hydrophobic interactions, proteins start to denature at room temperature above pressures of 100–200 MPa (Cheftel 1995). Oligomeric structures dissociate into their subunits, monomeric structures partially unfold and denature, and proteins aggregate and gel. The conformation of proteins is altered by an increase in pressure, due to irreversible changes to the secondary, tertiary, quaternary and supramolecular structures (Palou et al. 1999). Denaturation may result when proteins are exposed to pressure beyond that of the individual protein-specific pressure threshold (Cheftel 1995). The structure and function of lipids and polysaccharides are altered by HPP (Ledward 1995). However, pressure effects on lipids are usually reversible, which is often not the case for polysaccharides and proteins. Smaller molecules such as vitamin C and ß-carotene are not unaffected (Bull et al. 2004; Cheftel 1995). Oxidative reactions in foods and enzymatic browning in some fruits are reportedly enhanced by HPP, while partial discoloration has been reported in treated red meats (Cheftel 1995; Ledward 1995).

2.1.1. Pressure Effects on Viruses

The work of Giddings et al. (1929) was the first documented attempt to estimate the pressure sensitivity of viruses by studying tobacco mosaic virus (TMV). TMV was found to be extremely resistant to pressure; pressurization at 920 MPa was necessary to show any measurable inactivation. Fortunately, the pressure resistance of most human and animal viruses is lower than that of TMV. Most of these viruses can be inactivated at pressures <450 MPa (Table 15.1).

Nakagami et al. (1992) found pathogenic herpes simplex type 1 virus and human cytomegalovirus to be eliminated after 10 min exposures above 300 MPa at 25 °C. Kingsley et al. (2002) found that 450 MPa for 5 min at ambient temperature reduced hepatitis A virus in tissue culture medium to nondetectable levels from initial concentrations of 10^7 infectious units. For the human and animal viruses studied thus far, poliovirus appears to be the most resistant to pressure, capable of surviving an hour at 600 MPa with only modest reductions in infectivity (Wilkinson et al. 2001).

The extent of virus inactivation is dependent upon treatment pressure duration and temperature. Usually, the degree of virus inactivation is more dependent on variations in treatment pressure than duration, as was indicated by Jurkiewicz et al. (1995) who studied the pressure sensitivity of simian immunodeficiency virus (SIV). The infectivity of SIV was reduced by 5-$\log_{10}$ infectious units after a 1-h exposure to 250 MPa at 21.5 °C. Treatments at 200 and 150 MPa required 3 and 10 h, respectively to attain equivalent reductions of 5-$\log_{10}$ infectious units.

A number of reports have indicated the dissociation and denaturation of proteins and inactivation of viruses by pressure is promoted by low temperatures (Bonafe et al. 1998; Foguel et al. 1995; Gaspar et al. 1997; Kunugi and Tanaka 2002; Tian et al. 2000; Weber 1993). The explanation for this phenomenon is that low temperatures promote the exposure of nonpolar side chains to water. The nonpolar interactions are more affected by pressure because they are more compressible. Oliveira et al. (1999) examined the combined effect of pressure and low temperature on the stability of foot-and-mouth disease virus (FMDV), an animal virus that can cause devastating losses in the meat and dairy industries. FMDV was found to be sensitive to pressure; exposure to 240 MPa for 2 h resulted in loss of infectivity of 4-$\log_{10}$ infectious units at room temperature and 6-$\log_{10}$ units at –15 °C.

The effect of treatment temperature on the inactivation of λ phage, an *E. coli* phage, under high pressure was studied by Bradley et al. (2000). A 3- to 4-$\log_{10}$ decrease in titer was observed when λ phage in human plasma was pressurized at 275 MPa for 7.5 min at temperatures ranging from 62 to 44 °C; however, below –30 °C, the phage was only slightly inactivated by pressure, suggesting that there exists an optimal temperature for pressure inactivation of λ phage.

Rotavirus titer was found to decline by 5-$\log_{10}$TCID$_{50}$/ml within a 70 s exposure to 300 MPa at 25 °C, but 1-$\log_{10}$TCID$_{50}$/mL remained after a 10 min treatment (Khadre and Yousef 2002). Herpes simplex virus and human cytomegalovirus could not be recovered following 10 min of treatment at the

Table 15.1 Pressure inactivation of different viruses

Name	*Enveloped*	*Pressure (MPa)*	*Time (min)*	*Temperature (°C)*	*Loss in infectivity (log_{10})*	*References*
Aichivirus A846/88	No	600	5	21	No reduction	Kingsley et al. (2004)
Coxsackie virus A9	No	500	5	21	7	Kingsley et al. (2004)
Coxsackie virus B5	No	600	5	21	No reduction	Kingsley et al. (2004)
Feline calicivirus	No	275	5	22	7	Kingsley et al. (2004)
Foot and mouth disease virus	No	240	120	–15	6	Oliveira et al. (1999)
Hepatitis A virus	No	450	5	22	7	Kingsley et al. (2005)
Herpes simplex virus type 1	Yes	400	10	25	7	Nakagami et al. (1992)
Human cytomegalovirus	Yes	300	10	25	4	Nakagami et al. (1992)
Human immunodeficiency virus	Yes	350	10	25	>3	Nakagami et al. (1996)
Human parechovirus-1	No	500	5	21	4	Kingsley et al. (2004)
Infectious bursal disease virus	No	230	120	0	5	Tian et al. (2000)
Phage λ	No	400	20	22	7	Chen et al. (2004)
Poliovirus	No	600	60	20	<1	Wilkinson et al. (2001)
Rotavirus	No	300	2	25	8	Khadre and Yousef (2002)
Simian immunodeficiency virus	Yes	250	60	22	5	Jurkiewicz et al. (1995)
Sindbis virus	No	250	480	Not specified	5	Gaspar et al. (1997)
Vesicular stomatitis virus	Yes	260	720	20	4	Silva et al. (1992)

same pressure. These enveloped viruses were prevented from binding to host cells and subsequently initiating infection as a result of damage to viral envelopes sustained by HPP.

Most of the early HPP studies were conducted using feline calicivirus (FCV) as a surrogate for HuNoV. A 7-$\log_{10}$TCID$_{50}$/mL culture of FCV was completely inactivated in isotonic tissue culture medium after a 5 min exposure to 275 MPa or more (Kingsley et al. 2002), indicating applicability of HPP for inactivating HuNoV, but this surrogate cannot be relied upon to guarantee the susceptibility of HuNoV to the process. For example, hepatitis A virus (HAV) and poliovirus (PV) are both members of the picornavirus family, but have largely differing susceptibilities to HPP.

HPP studies on HAV, FCV, and PV were reported by Grove et al. (2008) demonstrating that various viruses react differently to HPP. HAV was inactivated by >1-$\log_{10}$TCID$_{50}$/mL and >2-$\log_{10}$TCID$_{50}$/mL after 600 s treatments with 300 and 400 MPa, respectively, and was undetectable (>3.5-$\log_{10}$TCID$_{50}$/mL reduction) within a 300 s treatment with 500 MPa. FCV was inactivated by 3.6-$\log_{10}$TCID$_{50}$/mL after a 120 s treatment with 300 MPa, and was undetectable after a 180 s treatment with 300 MPa. PV was the most resistant with little or no substantial reduction in titer after a 300 s treatment with 600 MPa. HAV was also found to be susceptible to HPP in contaminated strawberry puree and sliced green onions (Kingsley et al. 2005).

Increased salinity has been found to protect HAV from high pressure. The pressure required to inactivate HAV within 5 min increased when treated in seawater of 27.4 g/L salinity, as compared to an isotonic tissue culture medium (Kingsley et al. 2002). Salt may act to stabilize viral capsid proteins at high pressure, an observation which may have important implications for future applications of HPP to shellfish products. This observation was similarly described by Grove et al. (2009) and HPP inactivation data obtained for HAV was used to develop Weibull and log-linear models to predict inactivation. The models were evaluated by using high pressure to treat HAV artificially inoculated into Pacific oyster (*Crassostrea gigas*) homogenate adjusted to 15 or 30 g/L salinity. The log-linear model generally provided fail-safe predictions at pressures of 375 MPa and may aid shellfish processors wishing to incorporate HPP into an oyster processing regime.

2.1.2. Comparison of HPP Inactivation of Various Human Norovirus Surrogates

As described above, there are a wide variety of experimental and processing conditions used to evaluate various viruses and viral surrogates that makes comparison of inactivation results a very difficult task. Because different inactivation conditions may impact virus inactivation by HPP (Kingsley 2013), Cromeans et al. (2014) used the identical HPP test conditions (100–800 MPa) for several viruses and HuNoV surrogates and compared inactivation results. Aichivirus was stable at 800 MPa at 4 °C for 1 min; a similar result was

observed by Kingsley et al. (2004), where Aichivirus was stable at 600 MPa for 5 min at 4–21 °C. Inactivation of feline calicivirus (FCV) and MNV-1 was achieved at roughly similar pressures as reported previously (Chen et al. 2005; Kovac et al. 2012; Lou et al. 2011a, b). MNV-1 was more resistant than FCV, which was completely inactivated at 300 MPa. MNV-1 required 400 MPa and above for complete inactivation. Kingsley (2013) also found MNV-1 to be more resistant than FCV. Using HPP, an approximate 4-$\log_{10}$ reduction was reported for Tulane virus (TuV) and a 2-$\log_{10}$ reduction for MNV-1 in culture medium at 21 °C for 2 min using 350 MPa (Li et al. 2013). Cromeans et al. (2014) reported a 3.5-$\log_{10}$ reduction of each virus after exposure for 1 min at 4 °C using 300 MPa.

These differences clearly demonstrate the challenges in comparing viral inactivation results, particularly research conducted with the various cultivable surrogate viruses. It is possible that differences in temperature (e.g. the use of 4 and 21 °C for HPP studies) may contribute to different findings. However, the use of appropriate pressures are critical for successful inactivation of HuNoV by HPP, as was shown in a study where human volunteers were fed raw oysters which had been artificially seeded with Norwalk virus and treated with HHP (Leon et al. 2011). In this study, 600 MPa treatment resulted in complete inactivation of the virus and resulted in no human infections. Cromeans et al. (2014) also concluded that TuV and MNV-1 were the most resistant cultivable surrogate viruses and the best candidates to study public health outcomes of human norovirus infections.

2.1.3. Oyster and Bivalve Mollusks Processing

The application of HPP to whole oyster processing has been attractive for a variety of reasons. Oysters (and other shellfish) are high-value foods traditionally consumed raw throughout the world (Kingsley et al. 2005). Pathogens associated with raw oysters, notably *Vibrio* spp. and hepatitis A virus, are sensitive to inactivation by HPP (Calci et al. 2005; Kingsley et al. 2005; Styles et al. 1991). The refrigerated shelf-life of harvested oysters is limited, so any extension of the shelf-life without altering sensory quality is highly desirable. An extension of oyster shelf-life can be achieved by pressure treatment. Additionally, Lopez-Caballero et al. (2000) described pressure-treated oysters as 'slightly more voluminous with a very pleasant appearance', and reported that oysters were more appealing following treatment at chilled temperatures than at room temperature and above. Flavor may also be enhanced, possibly by pressure infusion of the salty liquor within the oyster shell into the meat (Hoover et al. 1989). Oysters are killed by high pressure treatment (McDonnell et al. 1995), and the adductor muscles holding the shell tightly closed are cleaved from the shell. As a result, pressure treatment ensures convenient manual shucking of the whole oyster without the need for shucking knives (Kingsley et al. 2005). This allows for higher yields, as there is both a full release of the muscle from the shell and no damage to the tissue

from the shucking knife. At 275 MPa, nearly 100 % of whole shell oysters are open. Usually a hold time of 1–2 min is used. Once shucked, the oyster meat can be manually shaken off the shell and further processed in semi-rigid containers at 415 MPa for several minutes, which extends the refrigerated shelf-life to three weeks (Farkas 2005).

One of the definitive studies that demonstrated the efficacy of HPP for inactivating viruses in shellfish and the first demonstration of virus inactivation by HPP in a human challenge study was demonstrated by Leon et al. (2011). A randomized, double-blinded clinical trial was conducted on Norwalk virus (HuNoV genogroup I.1) seeded oysters processed by HPP at various pressures and temperatures for 5 min and consumed by healthy volunteer adults. Oysters treated at 600 MPa at either 6 or 25 °C were completely inactivated and resulted in no virus infection in volunteers. Virus infection and viral genomic material were detected from volunteers who consumed oysters treated at 400 MPa. The authors noted that the number of volunteers infected in the group which consumed the 400 MPa-treated oysters was higher for oysters processed at 25 °C (60 %) than oysters processed at 6 °C (21 %). This study supports the effectiveness of HPP at inactivating HuNoV and while oysters processed at 600 MPa did not cause any human infection, the authors noted that 600 MPa-treated oysters were less visually acceptable with a cooked appearance. The study also supported results of earlier studies, which demonstrated that lower processing temperatures resulted in better viral inactivation and that more research is required to further optimize the process of inactivating viruses at lower pressures in order to maintain the quality of oysters.

2.2. Irradiation

While food irradiation has been shown to be an effective nonthermal means of preserving foods for the marketplace, its effectiveness against viruses is dependent on the size of the virus, the suspension medium, product characteristics, and the exposure temperature (Farkas 1998; Patterson 1993). Most viruses are far more resistant to irradiation than vegetative bacteria, parasites, or fungi which may be due in part to their smaller size and even smaller genome size (often single-stranded RNA) (Farkas 1998). Additionally, the treatment levels necessary to minimize organoleptic deterioration in irradiated foods has little effect on viruses, thus rendering irradiation impractical for the inactivation of viruses in commercial food products. The resistance of viruses to irradiation is comparable to that of bacterial endospores and extremely resistant vegetative types. For example, Monk et al. (1995) summarized resistances in D_{10} values (kilograys, kGy), the amount of irradiation required to reduce a microorganism population by 90 %, for important food microorganisms. Viruses had D_{10} values ranging from 2 to 8 kGy, in comparison to varieties of *E. coli* with D_{10} values of ~0.3 kGy. Radiation-resistant bacterial spores had D_{10} values of 2–10 kGy and the supremely resistant *Micrococcus radiodurans* (now *Deinococcus radiodurans*) showed D_{10} values of 12–14 kGy.

The efficacy of using gamma irradiation in combination with a sanitizer such as chlorine was demonstrated by Foley et al. (2002) where chlorination in combination with 0.55 kGy of irradiation resulted in a 5.4-$\log_{10}$ reduction of *E. coli* O157:H7 on shredded iceberg lettuce. Irradiation applied at that level was also sufficient in inactivating yeast and molds without causing softening of lettuce and no impact on the sensory attributes.

After studying the safety of irradiating fresh iceberg lettuce and fresh spinach, and determining these products retained nutrient value and are safe for consumers to consume, on August 22, 2008, the US FDA published the final rule that allowed the use of irradiation, with a maximum absorbed dose of 4.0 kGy, to ensure the safety of such products (FDA 2008b). It must be noted that irradiation is a complement to and not a replacement for proper food handling practices. Also, it is an additional tool that can be used to reduce the level of microbial pathogens in iceberg lettuce and spinach, but does not take the place of washing.

Another example of non-thermal technology is non-thermal plasma (NTP), an emerging sanitizing technology, which has recently been shown to be promising as an effective means for rapidly reducing pathogens at the surface of fresh produce and raw foods and may be classified as a form of irradiation. Recent research at one of the institutes applying this technology showed that levels of *Salmonella* and *E. coli* O157:H7 were reduced on the surface of apples by 2.9–3.7 $\log_{10}$ CFU/ml and 3.4–3.6 $\log_{10}$ CFU/ml, respectively, after a 3 min of treatment (Niemira and Sites 2008), and the bacteria were reduced on the surface of almonds by as much as 1.34 $\log_{10}$ CFU/ml in 20 s (Niemira 2012). NTP processes use high-voltage electricity to ionize gases near the electrodes of the device. Ionized gases, such as reactive oxygen species, are diffused toward the surface under the influence of electrical fields resulting in a rapid bactericidal effect. The antimicrobial efficacy of NTP is related to the specific design of the technology used, the power level used to generate the plasma, the gas composition and flow rate used in the plasma emitter, and the time and intensity of exposure.

2.3. Pulsed Electric Field

Pulsed electric field (PEF) can be used as a food processing tool for the destruction of microorganisms in liquids and pumpable foods (Sitzmann 1995). Food is pumped through a treatment chamber between positive and negative electrodes subjected to electric fields generated in time pulses ranging from a few microseconds to milliseconds (Stewart and Cole 2001). The process is continuous, and the fluids must be aseptically packaged immediately following treatment to prevent post-process contamination (Dunne and Kluter 2001). Examples of foods that may be treated with PEF include fruit juices, milk, liquid egg and soups (Vega-Mercado et al. 1999), pasta sauces, tomato salsas, and various yogurt-based foods (Dunne and Kluter 2001). PEF increases bacterial and fungal membrane permeability by destabilizing membrane proteins and the lipid bilayer structure, thus forming transmembrane

pores (Castro et al. 1993; Zimmermann 1986; Zimmermann and Benz 1980); however, to date, PEF has shown little effectiveness against viruses. Rotavirus is resistant to PEF (Khadre and Yousef 2002). PEF may be less effective against protein capsids as compared to lipid-rich membranes.

2.4. High-Intensity Pulsed Light

High-intensity pulsed light involves electrical ionization of a xenon lamp to emit a broadband white light with a spectrum resembling that of sunlight and containing wavelengths that include a large component (45 %) of UV light; UV irradiation will inactivate viruses (Nuanualsuwan and Cliver 2003). The intensity of pulsed white light required to inactivate viruses is estimated to be about 20,000 times the intensity of sunlight. Roberts and Hope (2003) investigated the potential of high intensity broad-spectrum white light to inactivate viruses. Enveloped viruses (e.g., Sindbis and herpes simplex virus type 1) and non-enveloped viruses (e.g., encephalomyocarditis, polio virus type 1, hepatitis A, bovine parvovirus, and canine parvovirus) were diluted in phosphate-buffered saline and placed in small plastic sample dishes at a depth of 5 mm. A dose of 1.0 J/cm^2 was found sufficient to inactivate 4.8–7.2 $\log_{10}$ of these viruses.

The efficacy of pulsed UV light for decontamination of minimally processed vegetables was investigated by Gomez-Lopez et al. (2005). A sensory evaluation was conducted with a semi-trained panel of 4–6 people who gave pulsed UV light-treated iceberg lettuce better scores than the control samples for off-odor, taste, and leaf edge browning. This clearly indicates that pulsed UV light treatment helps in preserving the lettuce quality. In general, pulsed UV light treatment of food may not cause any adverse effect if applied in moderate amounts. This is required for microbial inactivation as strongly suggested by previous studies. However, modification and optimization of the treatment may be necessary for successful implementation of the process in some foods.

Gomez-Lopez et al. (2005) obtained 0.21- and 1.67-$\log_{10}$ reductions after treating minimally processed vegetables, such as spinach, celery, green paprika, soybean sprouts, radicchio, carrot, iceberg lettuce, and white cabbage, with 7 J pulsed UV source for 45 s/side. Hoornstra et al. (2002) reported that carrots and paprika treated with two pulses of pulsed UV (0.30 J/cm^2) resulted in reductions of 1.6 $\log_{10}$ CFU/cm^2 and >2.6 $\log_{10}$ CFU/cm^2, respectively, in the total aerobic count on the surfaces, respectively. The authors reported no adverse effect on vegetables quality when stored at 7 or 20 °C for up to 7 days. Sharma and Demirci (2003) obtained more than a 4 $\log_{10}$ reduction of *E. coli* O157:H7 when inoculated alfalfa seeds were treated with pulsed UV.

Bialka and Demirci (2007) treated blueberries with pulsed UV for inactivation of *E. coli* O157:H7 and *Salmonella* Typhimurium. They observed maximum reductions of 4.3 and 2.9 $\log_{10}$ CFU/g for *Salmonella* and *E. coli* O157:H7, respectively, with a 60 s treatment at 8 cm from the pulsed UV source. Pulsed UV did not change the sensory or color attributes of the blueberries.

Similarly, *E. coli* O157:H7 and *Salmonella* Typhimurium populations were reduced by 3.9 $\log_{10}$CFU/g and 3.4 $\log_{10}$CFU/g at 72 and 59.2 J/cm^2, respectively, in raspberries (Bialka and Demirci 2008). When strawberries were treated with pulsed UV at 25.7 and 34.2 J/cm^2, maximum reductions of 2.1 and 2.8 $\log_{10}$CFU/g, were obtained for populations of *E. coli* O157:H7 and *Salmonella* Typhimurium, respectively. The authors noted that there was no observable damage to the fruits due to pulsed UV treatment.

Vimont et al. (2015) demonstrated the exposure of MNV-1 to 12 J/cm^2 of pulsed light in various liquid matrices (buffered saline, hard water, mineral water, turbid water and sewage effluent) and on various food contact surfaces (high density polyethylene, polyvinyl chloride and stainless steel) was sufficient to inactivate >3 $\log_{10}$ MNV-1 within 3–6 s depending on the clarity of the water or cleanliness of the surfaces. Vimont et al. (2015) elucidated that the mechanism involved in the antiviral activity of pulsed light was probably due to the disruption of MNV-1 viral structure that ultimately degraded viral proteins and RNA.

Liu et al. (2015) used a novel experimental set up of incorporating water assisted UV processing to inactivate MNV-1 artificially inoculated onto the surfaces of blueberries and showed that with exposure of 12,000 J/m^2, MNV-1 reductions of >4.32 $\log_{10}$ were achieved with water assisted UV-processing as compared to the 2.48 $\log_{10}$ reduction obtained with dry UV treatments. Liu et al. (2015) further indicated the combination of water assisted UV processing had better or similar inactivation over the use of chlorine (10 ppm) alone. The application of UV and the sequence in which it is applied could impact viral inactivation. In a study by Rattanakul et al. (2015), the combination of UV (up to 50 mJ/cm^2) and chlorine (up to 0.15 ppm) applied either simultaneously or sequentially produced greater inactivation of human adenovirus suspended in buffer. When chlorine was applied sequentially before UV treatment, inactivation rates of human adenoviruses were higher than when sequential application of UV were followed by chlorine. This is possibly because of the sensitivity of human adenovirus to chlorine and better disruption of the viral capsid leading to better UV-induced damage of the viral genome through creation of pyrimidine dimers, thereby interrupting polymerase activity during the replication process.

The efficacy of UV or pulsed light systems as a wash water disinfectant can be impacted by the turbidity of the wash water due to the limited penetration capacity of UV. Their application in wash water systems could be limited to filter disinfection or irradiation of recirculating water streams. Since there is a required exposure time for UV to act on microorganisms and there is commonly a fast flow rate in wash systems, not all microorganisms may be inactivated when passing through the UV source. However, UV or light systems could be incorporated onto conveyer belt systems or sorter systems prior to the product entry into the wash flume to reduce the microbial load on the product and enhance the efficiency of the disinfectant in the wash flume.

2.5. High Power Ultrasound

Bacterial cells and viruses can attach to all surfaces of leafy green vegetables. If attached to freshly cut surfaces that are leaking nutrients or in pores or stomata, the ability of the antimicrobial sanitizer will be diminished (Takeuchi and Frank 2000). Physical methods of removing bacterial cells and viruses are important, and shear forces generated during washing may assist in dislodging foreign particles into the sanitized water.

High power ultrasound (HPU) has been used for decades in various industries for cleaning purposes and mounting evidence suggests that incorporating HPU into commercial produce washing lines can assist in removing bacterial cells and virus particles (virions) from produce surfaces or by improving the access of the chemical sanitizers to the attached microorganisms.

HPU creates a partial vacuum in the liquid that forms tiny bubbles which rapidly collapse under the vacuum. The bubbles collapse thousands of times each second, producing high-energy shockwaves that travel through the liquid. The shockwaves have the capacity to scour contamination and microorganisms from surface cracks and pore spaces and from internal cellular structures of agricultural products, which under conventional cleaning and sanitation processes would be difficult to remove. This mechanism accelerates physical and chemical reactions to enhance processes such as cleaning, plastic welding, and sonochemistry. Ultrasound technology is easily adaptable to existing washing systems in industry, such as flumes and troughs and can be used in combination with existing approved sanitizers. The combined technology of HPU with existing approved sanitizers can be developed in a number of ways for the agriculture industry to bring improved food safety, increased shelf life, and improved food quality to commercial products. The additional benefit of HPU technology in produce washing is the ease with which it may be installed into existing washing flumes and the impact it may have on water reuse and conservation.

Maks et al. (2009) reported the effectiveness of chemical sanitizers to reduce the levels of pathogenic microflora was enhanced by 1–2 $\log_{10}$ after the addition of high power ultrasound (HPU) in the washing of fresh-cut lettuce. The authors demonstrated an additional 1-$\log_{10}$ reduction of total bacterial microflora on fresh cut lettuce leaves with the combined use of HPU with peroxyacetic acid (POAA), as compared to the sanitizer alone. Liu et al. (2009) demonstrated treatments with 25 or 100 ppm sodium hypochlorite reduced infectious murine norovirus (MNV-1) titers by 1.7 and 2.3 $\log_{10}$, respectively, and the addition of HPU during treatment increased reduction to 2.7 and 3.1 $\log_{10}$, respectively. Similar reductions were recorded for treatments with 80 ppm POAA, with a 2.5-$\log_{10}$ reduction achieved alone with POAA and a 3.7-$\log_{10}$ reduction achieved when combined with HPU and POAA (Fig. 15.1). Seymour et al. (2002) reported that inactivation of *Salmonella* Typhimurium by a combined HPU and chlorinated wash water treatment was enhanced by

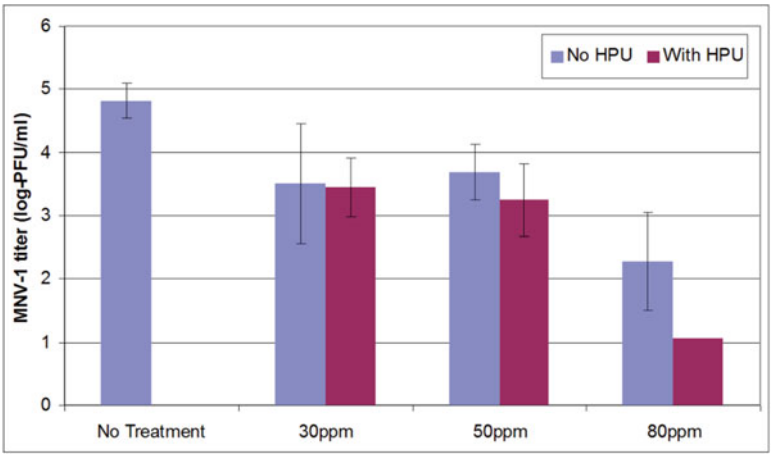

Figure 15.1. Effect of POAA on MNV-1 attached to romaine lettuce pieces treated with dH_2O or POAA (30, 50 and 80 ppm) with and without HPU at 10 °C (Liu et al. 2009).

1 $\log_{10}$, from a 1.7-$\log_{10}$ reduction with chlorinated water alone, to a 2.7-$\log_{10}$ reduction achieved with the combined treatment. Zhou et al. (2009) demonstrated an additional 0.7–1.1 $\log_{10}$ reduction of *E. coli* O157:H7 from the surface of spinach leaves with the combined treatment as compared to the use of water or various sanitizers alone.

There has been a trend of replacing chlorine with other alternate sanitizers because of the concerns about its efficacy on the product and the environmental and health impacts associated with the formation of halogenated by-products (Olmez and Kretzschmar 2009). Commercial post-harvest washing of fresh-cut leafy green vegetables are performed with water containing antimicrobial sanitizers in order to prevent cross-contamination of pathogenic microorganisms between contaminated leaves and uncontaminated leaves (FDA 2008a). A substantial body of research is available to suggest that while cross contamination may be prevented by commercially available sanitizers, these are generally ineffective at reducing microbial counts from the surface of fresh-cut produce by more than 3-$\log_{10}$ (Beuchat et al. 2004; Lou et al. 2011a, b; Beuchat and Ryu 1997; Gil et al. 2009; Delaquis et al. 2002). Sanitizer effectiveness also tends to decrease in the presence of organic materials, such as those often found in the commercial wash tanks of fresh produce items (Poschetto et al. 2007; Nou and Luo 2010; Grove et al. 2008). This has led to interest in the fresh produce industry for alternative sanitizers that are not affected dramatically by organic material and can achieve significant inactivation of pathogenic microorganisms that may be present.

3. SANITIZERS USED IN FOOD PROCESSING

Since there are no practical technologies that provide a kill step in eliminating pathogens from fresh fruits and vegetables without sacrificing product quality and shelf life, potential sources of contamination from the environment to the consumer's table should be identified and specific measures or mitigation steps taken or implemented to minimize the risk associated from such products. The application of good hygienic practices during production, transport, and processing, in combination with Good Agricultural Practices, Good Manufacturing Practices, Hazard Analysis Critical Control Point systems and using validated systems with appropriately verified monitoring controls will certainly minimize the contamination of fruits and vegetables and significantly reduce the risk of illness associated with the consumption of such products.

The act of washing fruits and vegetables using potable water followed by a rinse step in potable water would assist in the removal of microorganisms from the surfaces of these products. The incorporation of an appropriate sanitizer could enhance the removal of microorganisms by 10- to 100-fold. However, the efficacy of the sanitizer or disinfectant in relation to the physical properties of fruits and vegetables require further study since the effectiveness of the sanitizer can be affected by factors such as pH, contact time, organic load, temperature and water properties, in addition to the physical properties of the fruit and vegetable being washed.

The legal use of various sanitizers or disinfectants differs from country to country. In the USA, wash water sanitizers or disinfectants used for fresh-cut produce are regulated by the FDA as a secondary direct food additive, unless the sanitizers are considered to be Generally Recognized As Safe (GRAS). If the product is a raw agricultural commodity that is washed in a food processing facility, such as a leafy green washing facility, both the US Environmental Protection Agency (EPA) and the FDA have regulatory jurisdiction and the sanitizer must be registered with the EPA as a pesticide. A list of approved solutions for use as sanitizers or disinfectants can be obtained from the Code of Federal Regulations (CFR) 21, Sections 173.315 and 178.1010. The FDA is recommending a 5-$\log_{10}$ reduction of pathogenic microorganisms on produce (FDA 1995).

A number of new and novel sanitizers have emerged over the last decade that had various effectiveness on various microbial pathogens, but for the scope of this chapter, only sanitizers that have microbial testing data or are commonly used by the food industry will be addressed. This list is by no means exhaustive.

3.1. Chlorine

Chlorine and hypochlorites have been used for many years as a wash or spray sanitizer or in flume waters in the fresh produce industry. They are also often applied to food contact surfaces and processing equipment. Chlorine is commonly used at concentrations ranging from 50 to 200 ppm or mg/L with typical contact times of 1–2 min. However, chlorine concentrations can rapidly

decrease when chlorine comes into contact with organic matter or when it is exposed to air, light and metals. Prolonged exposure to chlorine vapors during preparation and mixing of the sanitizer can cause irritation to the skin and respiratory tract and pose potential occupational issues to workers in that environment.

Chlorine is a low-cost sanitizer with an established ability to kill pathogens in suspensions. This ability is largely due to the formation of hypochlorous acid as free chlorine (free active chlorine), which is the most effective form of chlorine (Zagory 1999). Maintaining the appropriate amount of free chlorine in a wash flume setting can be technically challenging and the amount of free chlorine, like any other oxidant antimicrobial agents, is impacted by pH, temperature and the amount of organic matter in the system (Zagory 1999; Gil et al. 2009). Similarly, organic latex or vegetable exudates released into the wash flume from wounded tissues or cut surfaces can significantly impact chlorine effectiveness. This is particularly challenging especially at the front end of the wash flume where large quantities of produce are continuously added and the surge of organic matter and exudates can rapidly deplete free chlorine, creating a situation where the demand for free chlorine exceeds its availability and thereby increases the risk for cross-contamination or transfer of pathogens to uncontaminated product. The minimum amount of free chlorine that is effective in inactivating suspensions of *E. coli* O157:H7 to a non-detectable level has been shown to be around 0.5 mg/L or ppm, but this level could quickly increase to 10 mg/L or ppm or higher levels of free chlorine when in the presence of product in the wash water before washing (Lou et al. 2011a, b). Repeated addition of chlorine to wash water that is high in organic load may be counterproductive as this results in the increased formation of toxic chlorine by-products and generation of harmful chlorine off-gas (Suslow 2001).

The porcine gastric mucin binding magnetic bead (PGM-MB) assay was used by Kingsley et al. (2014) as a screening tool for estimating human norovirus infectivity and to determine the effects of various chemical sanitizers to inactivate human norovirus in a stool suspension. A 1-minute treatment using chlorine concentrations of up to 189 ppm reduced PGM-MB binding by up to 4.14 $\log_{10,}$ which was estimated to equate to reduction of human norovirus infectivity. The data suggests the use of chlorine, at appropriate concentrations, is still one of the most efficient sanitizers in inactivating human noroviruses.

In order to minimize the quick depletion of free chlorine by organic loads in the wash flume, free chlorine stabilization has been researched, much like the use of cyanuric acid in maintaining free chlorine levels in outdoor swimming pools (Sommerfeld and Adamson 1982), where formation of a relatively stable compound, chlorimide, is more resistant to degradation. Nou et al. (2011) explored the use of a proprietary formula named T-128 in stabilizing free chlorine in the presence of high organic load and evaluated its impacts on the quality and shelf-life of fresh-cut iceberg lettuce.

Nou et al. (2011) reported that T-128 significantly reduced the rate at which free chlorine was depleted in the presence of soil in the wash system and that T-128 also significantly reduced the survival of bacterial pathogens in the wash

system with high organic loads, thereby potentially reducing the risk of cross-contamination when contaminated and uncontaminated produce were co-washed in the same system. The application of T-128 in the wash system did not have significant impact on the quality and shelf-life of the produce being washed. However, the authors noted that T-128 did not enhance the efficacy of chlorinated wash solutions for microbial reduction on contaminated produce.

3.2. Organic Acid Based Sanitizers

Organic acids are naturally present in fruits and vegetables and organic acids accumulate in fermented products. They retard the growth of some microorganisms and prevent the growth of others. Typically, microbial pathogens do not grow in pH environments below 4.0 so acidic pH environments or portions of the fruit or vegetable generally would not be areas of proliferation for microbial pathogens. Organic acids such as acetic, citric, succinic, malic, tartaric, benzoic and sorbic acids have been applied in various applications to either control spoilage or pathogenic bacteria on various foods such as the application of lemon juice to control *Campylobacter* species populations in watermelon and papaya (Castillo and Escartin 1994).

Some of the early studies using organic acids were conducted by Shapiro and Holder (1960) where 1500 ppm of citric acid was used to treat salad vegetables and did not affect the growth of bacteria in a 4-day storage trial at 10 °C, but when the vegetables were treated with 1500 ppm of tartaric acid, a tenfold reduction in total counts was observed. Karapinar and Gonul (1992) used a solution containing 2 % acetic acid or 40 % vinegar to wash parsley that was artificially inoculated with 7-$\log_{10}$ of *Yersinia enterocolitica* and after the 15 min washed, <1-$\log_{10}$ of *Y. enterocolitica* was detected. A 30 min wash with 5 % acetic acid resulted in no detectable bacteria.

One common example of an organic acid-based sanitizer is peroxyacetic (peracetic, POAA) acid. Peroxyacetic acid is a strong oxidizing agent that has been used extensively for processing equipment sanitization and is FDA approved (up to 80 ppm) for applications on fruits and vegetables (FDA 2015). A number of studies, such as those of Simons (2001) and Gonzalez et al. (2004), demonstrated POAA efficacy in pilot-scale or simulated wash conditions on minimally processed produce against *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp. In these studies, up to a 2-$\log_{10}$ reduction of inoculated pathogens on produce items such as carrot shreds was observed and POAA was not as affected by the presence of organic matter when compared to chlorine.

Gulati et al. (2001) and Baert et al. (2009) demonstrated the efficacy of POAA on viruses inoculated onto various produce items. Gulati et al. (2001) showed a 1- and 2-$\log_{10}$ reduction of FCV on strawberries and lettuce, respectively, when treated with 150 ppm POAA and no reduction was observed for a similar experimental set-up using 200 ppm chlorine. Allwood et al. (2004) showed comparable reductions of MS2 and FCV on lettuce using 80 ppm POAA. Baert et al. (2009) demonstrated that 250 ppm POAA was required to achieve a 1-$\log_{10}$ reduction of MNV-1 on shredded iceberg lettuce and its use

was more effective than a comparable 200 ppm chlorine where <1 $\log_{10}$ reduction of MNV-1 was observed. Kingsley et al. (2014) showed similar results, where 195 ppm of POAA with a 1-min treatment time on human norovirus stool suspension achieved a <1 $\log_{10}$ reduction.

3.3. Electrolyzed Water

Electrolyzed water (EW) has been shown as a viable alternate disinfection tool for wash water (Ongeng et al. 2006). Gil et al. (2009) listed the Ecodis® technology in their review article, citing the use of anodic oxidation principles in the technology which consists of a highly efficient electrolysis cell equipped with coated permanent titanium electrodes. A direct low-voltage current that passes across the electrodes in a NaCl solution causes the formation of potent oxidizing agents derived from oxygen and free chlorine when chloride ions are present in the wash water. The oxygen and chloride radicals react with each other to form hypochlorous acid and hypochlorite ions that may have residual activity after the product leaves the wash flume. Different forms of EW can exist—acidic EW with a typical pH of 2.2–2.4, neutral EW with a typical pH 7.0–8.0 and basic EW with a typical pH 10–11.

The anode end of the electrolysis process produces acidic EW with a low pH of around 2.5 or less and is reported to have strong antibacterial activity against a number of pathogens. The effects of acidic EW were demonstrated by a number of researchers to decontaminate surfaces of lettuce, tomatoes, strawberries, cucumbers and spinach (Koseki et al. 2001, 2004; Bari et al. 2003; Guentzel et al. 2008). Acidic EW was also effective for the inactivation of *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes* and *Bacillus cereus* on the surfaces of fresh produce as demonstrated by various researchers (Venkitanarayanan et al. 1999; Kim et al. 2000; Park et al. 2001; Park et al. 2008). However, the effectiveness of acidic EW can be impacted by organic matter, which will affect its ability to inactivate pathogens on surfaces of produce items (Park et al. 2008).

In another study conducted by Kim et al. (2006), acidic EW was used to treat alfalfa and broccoli seeds. The acidic EW treatment at 55 °C for 10 min, produced from an acidic EW generator using deionized water and 10 % NaCl solution resulted in a 3.4- and 3.3-$\log_{10}$ reduction of *E. coli* O157:H7 from alfalfa and broccoli seeds, respectively, and did not significantly impact the germination rates of the seeds.

The effects of EW are not as well described for viruses compared to bacterial pathogens. Different forms of EW and their effects on viruses have been studied by a handful of researchers. The use of acidic EW, which has been demonstrated to be effective on bacterial pathogen removal and inactivation, demonstrated the opposite effect by Tian et al. (2011) where acidic EW washes enhanced the binding of human noroviruses onto raspberries and lettuce, reducing viral removal from these food surfaces. However, Tamaki et al. (2014) demonstrated the presence of free chlorine, at a minimal concentration of 40 ppm, with neutral EW treatment inactivated avian influenza H5N1 viruses

by >5 $\log_{10}$ after a 1 min treatment. The use of acidic EW similarly inactivated avian influenza H5N1 viruses by >5 $\log_{10}$ after a 1 min treatment, but the effectiveness of acidic EW was not dependent on the availability of free chlorine (Tamaki et al. 2014).

Slightly acidic EW, with a pH 5.0–6.5, was shown to be a novel disinfectant for the inactivation of porcine reproductive and respiratory syndrome virus and pseudorabies virus (Hao et al. 2013). High levels of viral inactivation, 7.0 $\log_{10}TCID_{50}$/ml and 5.9 $\log_{10}TCID_{50}$/ml were achieved for porcine reproductive and respiratory syndrome viruses and pseudorabies viruses, respectively, in the presence of at least 50 ppm of free chlorine with a 10 min exposure.

It is possible that the effectiveness of EW on viruses could be dependent on the presence of free chlorine in the wash system. A recent study conducted by Fang et al. (2016) examined the effectiveness of acidic EO and neutral EO to inactivate MNV-1 and HAV and the impact of organic loads on EO effectiveness. This study highlighted that concentrations of chlorine, 30–40 ppm, were required to achieve a 3-$\log_{10}$ inactivation of MNV-1 and HAV and increasing concentration of chlorine increased the virucidal effects of acidic EO and neutral EO. However, the presence of organic loads in water significantly impacted the ability of acidic EO and neutral EO to inactivate MNV-1 and HAV. Acidic EO was also shown to be more effective than neutral EO and with increasing levels of organic loads, the authors noted a longer treatment time and higher levels of free chlorine were required to inactivate MNV-1 and HAV (Fang et al. 2016).

3.4. Chlorine Dioxide

Chlorine dioxide (ClO_2) is another promising non-thermal technology that can be applied either as a gas or liquid for the reduction of pathogens in fresh fruits and vegetables. When applied as a gas, ClO_2 at 4 ppm with an exposure time of 30 min could achieve a 5-$\log_{10}$ reduction of *E. coli* O157:H7 and *L monocytogenes* on strawberries (Han et al. 2004) and a 4.4-$\log_{10}$ reduction of *Salmonella* spp. on strawberries was observed after exposure to 8 ppm ClO_2 gas for 120 min (Sy et al. 2005). Similarly, the application of gaseous ClO_2 (4.1–8.0 ppm) to strawberry, blueberries and raspberries could have phytosanitary applications without impacting fruit quality because it is effective in inactivating yeasts and molds that could extend the shelf life of the product and provide opportunities to the industry to export to further markets (Sy et al. 2005).

Studies on other produce showed that treatment of uninjured green peppers with 3 ppm ClO_2 gas reduced populations of *L. monocytogenes* by more than 6 $\log_{10}$ after 30 min exposure (Han et al. 2001b). Additionally, Han et al. (2001a) found that treatments of uninjured green peppers with 0.6 ppm ClO_2 gas reduced populations of *E. coli* by 7.3 $\log_{10}$ after 30 min at 22 °C in a 90–95 % relative humidity environment. Du et al. (2003) reported that treatment of apples with 3 ppm ClO_2 gas for 20 min resulted in a 5.9-$\log_{10}$ reduction of *E. coli* after 30 min and treatments conducted with 4 ppm ClO_2 gas for 30 min reduced *L. monocytogenes* on apple pulp skin by 6.5 $\log_{10}$ (Du et al. 2002).

Chlorine dioxide, unlike traditional chlorine sanitizers, does not participate in chlorination reactions that result in harmful byproducts. Aqueous chlorine dioxide applied at 5 ppm was able to achieve a greater than 5-$\log_{10}$ reduction of *L. monocytogenes* and *E. coli* O157:H7 on apples, lettuce and cantaloupe (Rodgers et al. 2004). However, other studies have found that the same level of aqueous chlorine dioxide was only capable of reducing *L. monocytogenes* on lettuce by 1.7 $\log_{10}$ CFU/g (Zhang and Farber 1996). It is possible that such variability of results could be dependent on the method used to inoculate the produce (Rodgers et al. 2004).

Fresh-cut leaves of romaine or iceberg lettuce, inoculated by immersion in water containing 8 $\log_{10}$ of *E. coli* O157:H7 suspension for 5 min and dried in a salad spinner, were washed for 2 min in various concentrations (20–200 ppm) of aqueous chlorine dioxide which was effective in reducing *E. coli* O157:H7 populations on iceberg lettuce by up to 1.25 $\log_{10}$ and by approximately 1 $\log_{10}$ on romaine lettuce (Keskinen et al. 2009).

The use of chlorine dioxide and its effectiveness in inactivating viruses is not well characterized or widely investigated. Kingsley et al. (2014) used a PGM-MB assay to assess inactivation of human norovirus in 10 % stool filtrate and demonstrated that the use of 350 ppm chlorine dioxide dissolved in water was able to reduce human norovirus binding to PGM by 2.8 $\log_{10}$ with a 60 min treatment time. However, shorter exposure times, such as a 60 s treatment, did not reduce human norovirus binding to PGM, suggesting human norovirus was not inactivated.

4. SUMMARY AND CONCLUSIONS

The food safety hazards posed by human noroviruses are well documented. As the human population continues to grow, demand for all types of foods will continue to rise. Additional hurdle steps in food processing, such as implementation of an appropriate nonthermal processes into the production sequence, will reduce the risk of viral foodborne disease, but only in combination with good manufacturing and food hygiene practices.

Of all the non-thermal technologies explored, the application of HPP to commercial oyster processing appears to be a success story. HPP of shellfish provides a value-added product with the benefits of shelf-life extension (nearly double), an increased level of safety, and improved sensory quality generated by pressure-shucking of the shellfish. The capital cost of HPP equipment is high, and the cost of training employees in the operation of the equipment must be considered, but costs would be expected to drop somewhat due to the simplified shucking procedure and wider implementation of the technology in the food industry.

While processing technologies may improve the safety level of the product, it can never replace sound harvesting and manufacturing practices and common sense with regard to sanitation and hygiene. Incorporation of an

additional preservative step, such as HPP, to an existing process line should assist in the minimizing of microbial hazards from many foods including sea foods and minimally processed produce.

Similarly, sanitizer efficacy is affected by a number of factors such as organic loads, type of produce and other physiochemical properties that require careful evaluation and the maintenance of effective sanitizer concentrations (e.g. in the wash flume) so that wash water does not become a source of cross-contamination.

As with all new technologies, non-thermal processing technologies included, they may not be suitable to all applications and will require careful consideration. The use of these technologies to provide an additional hurdle to washing with a sanitizer is welcomed and the use of new processing technologies to enhance food safety must be cost-effective and have long-term sustainability. Intervention strategies that are developed to reduce and eliminate viruses must be flexible to serve both small and large establishments. These intervention strategies must also be affordable, effective and efficient. In all cases, validation studies conducted in large-scale trials using appropriate target pathogens in carefully planned challenge studies will provide additional knowledge and confidence that these intervention strategies are effective and scalable and bridge the gap between laboratory-scale experiments conducted in well controlled environments to pilot-scale or large-scale, real-life conditions.

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CHAPTER 16

Natural Virucidal Compounds in Foods

Kelly R. Bright and Damian H. Gilling

1. INTRODUCTION

Numerous plants have been shown to possess significant antibacterial, antifungal, antiviral, insecticidal, antioxidant, and anti-cancer properties (Didry et al. 1994; Friedman et al. 2002; Hammer et al. 2002; Knowles et al. 2005; Kordali et al. 2005; Peñalver et al. 2005; Carson et al. 2006; Pinto et al. 2006; Callaway et al. 2008; Ravishankar et al. 2008; Ravishankar et al. 2009; Reichling et al. 2009; Ravishankar et al. 2010). Natural antimicrobial compounds are produced in various parts of the plants e.g., flowers, buds, fruits, seeds, herbs, roots, leaves, bark, wood, and stem, coinciding with the various assaults that the plant might encounter in the environment (Burt 2004). The fragrance of plants is carried in the quinta essential, or essential oil fraction (Cowan 1999). These volatile oils are aromatic, viscous liquids (Burt 2004) that are complex mixtures of lipophilic and volatile secondary metabolites such as monoterpenes, sesquiterpenes, and/or phenylpropanoids. They are primarily responsible for a plant's fragrant and biological properties (Reichling et al. 2009).

Essential oils and other components may be separated from plants through processes such as extraction (liquid-liquid extraction, solid-phase extraction, supercritical fluid extraction, pressurized liquid extraction, microwave-assisted extraction, and ultrasound-assisted extraction), distillation, and cold pressing (Burt 2004; Rasooli 2007; Garcia-Salas et al. 2010). Of these, steam distillation is the most commonly employed commercial method for extraction of essential oils (van de Braak and Leijten 1999).

Essential oils are typically mixtures of many compounds and may have as many as 60 individual components (Senatore 1996; Russo et al. 1998). The composition can change depending on the geographical location, the soil, and even the season, leading to the biosynthesis of different metabolites. The active ingredient is often the dominant component, at times accounting for greater than 50 % of its chemical composition (Burt 2004). For instance, the carvacrol content of oregano oil may be as high as 85 % and the cinnamaldehyde content of cinnamon oil as high as 86 %, depending on their geographical origin (Ravishankar et al. 2009). Lemongrass oil contains multiple components including citral (57.5 %), citral diethylacetal (24.7 %), limonene (6.4 %), citral

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acetate (2.1 %), myrcene (1.2 %), and methyl heptenone (1.2 %) (Katsukawa et al. 2010). Eugenol is the primary active component in both clove bud oil (up to 85 %) (Farag et al. 1989; Bauer et al. 2001) and allspice oil (Takemasa et al. 2009) and accounts for their antioxidant properties (Ogata et al. 2000;Takemasa et al. 2009).

Many phytochemicals are routinely used in the average domestic kitchen cabinet. Their longstanding usage has resulted in many of these antimicrobials being considered as Generally Regarded as Safe (GRAS) compounds (Dillon 1999; Ress et al. 2003; Adams et al. 2004; Knowles et al. 2005). Plant extracts/essential oils have been used in many commercial applications e.g., to provide flavoring to foods and fragrances in perfumes. They have also been added to toothpastes, shampoos, ointments, and cosmetics (Burt 2004). Oregano oil has been used in salad dressings, tomato sauces, and pizzas (Ravishankar et al. 2009). Lemongrass is widely used as a food flavoring and fragrance component in perfumes and also for its analgesic and anti-inflammatory characteristics (Ress et al. 2003; Katsukawa et al. 2010). Cinnamon oil is also used as a flavoring agent in some foods (Friedman et al. 2002).

1.1. Types of Plant Antimicrobials

There are several groups of plant antimicrobials including saponins, thiosulfinates, glucosinolates, terpenoids, and polyphenols. Saponins are naturally occurring glycosides that may exhibit antimicrobial, hemolytic, membrane depolarizing, cholesterol binding, and allopathic activities (Naidu 2000). Thiosulfinates are related to garlic, which has been used as a medicinal plant since antiquity and possesses both static and cidal effects against microbes (Naidu 2000). Glucosinolates are bioactive compounds found in cruciferous vegetables such as cabbage and broccoli (Naidu 2000). Terpenoids are secondary metabolites and are highly enriched in compounds based on an isoprene structure (Cowan 1999). Citral is a type of terpenoid that causes bacterial membrane disruption and the leakage of intracellular ions. Its action on the cell membrane also has dramatic effects on proton motive forces, intracellular ATP content, and the overall cell activity (Somolinos et al. 2010).

Phenolic compounds are present in all plants (Bravo 1998). To date, more than 8000 phenolic compounds have been identified. The phenols and polyphenols are a group of bioactive chemicals which consist of a single substituted phenolic ring (Cowan 1999); different compounds vary in their C6 ring structure. In general, phenols or polyphenols exhibit the greatest antimicrobial efficacy of various plant origin compounds (Burt 2004). The antioxidant activity may occur via mechanisms such as scavenging of radicals and chelating of metal ions. The phenol, eugenol, reportedly participates in photochemical reactions (Mihara and Shibamoto 1982) and inhibits the production (Farag et al. 1989) and activity of enzymes (Wendakoon and Sakaguchi 1995). It may also cause changes in membrane permeability resulting from the exit of potassium ions (Walsh et al. 2003). Cell wall deterioration and lysis have also been observed (Thoroski et al. 1989).

Catechol and epicatechin are two forms of simple phenols (Peres et al. 1997; Toda et al. 1991). Phenolic acids, quinones, flavonoids, flavones, tannins (also called proanthocyanidins), and coumarins round out the class of phenolics. Red, blue, and purple berries, red and purple grapes, red wines, teas (especially green), apples, pears, raspberries, apples, onions, broccoli, soybeans, legumes, gingko biloba, and chocolate are common sources of polyphenols (Ullah and Khan 2008). Polyphenols exhibit anti-inflammatory, antimicrobial, anti-tumor, cardioprotective, and neuroprotective properties (Ullah and Khan 2008). Grape seed extract contains tannins, olive extract contains oleuropein, and green tea extract contains tannin and catechins. The high concentrations of epicatechin and catechin in grape seed extract and caffeic acid and epicatechin in green tea extracts account for their high antioxidant activities (Rababah et al. 2004).

Flavonoids are the most common polyphenolic compounds in plants (Kris-Etherton and Keen 2002) with over 4000 identified (Higdon and Drake 2005). They are found in numerous foods such as fruits (e.g., citrus, berries, apples), vegetables (e.g., onions), herbs, wines, teas, coffee, and chocolate (Higdon and Drake 2005). Flavonoids are considered secondary plant metabolites some of which are essential for plant function including roles in supportive tissues, plant defense strategies, and signaling properties (Garcia-Salas et al. 2010).

2. ANTIVIRAL ACTIVITY OF PLANT COMPOUNDS

While the antimicrobial properties of plant based essential oils have been examined in detail against bacteria (Didry et al. 1994; Friedman et al. 2002; Knowles et al. 2005; Peñalver et al. 2005; Callaway et al. 2008; Ravishankar et al. 2009; Reichling et al. 2009; Ravishankar et al. 2010), they have only recently been examined for their antiviral properties. The majority of this work has been directed towards enveloped viruses of clinical importance (Reichling et al. 2005; Koch et al. 2008; Loizzo et al. 2008; Meneses et al. 2009; Reichling et al. 2009; Garcia et al. 2010; Jackwood et al. 2010; Ocazionez et al. 2010; Wu et al. 2010; Garozzo et al. 2011), while limited research has been done on the efficacy of essential oils against non-enveloped viruses. In general, non-enveloped viruses are more resistant to environmental conditions and the action of antimicrobials than enveloped viruses (Watanabe et al. 1989; Barker et al. 2001).

It is impossible to describe every scientific paper examining the antiviral properties of plant compounds and hence the focus of this chapter is to provide the reader with examples of the types of studies that have been performed to date (e.g., the types of antimicrobials, the viruses tested); the results of such studies (e.g., whether or not the compound had antiviral activity against different types of viruses, namely viruses classified in different families or groups,

enveloped or non-enveloped viruses, DNA or RNA viruses, and viruses causing respiratory or gastrointestinal symptoms, etc.); and to point out any clues as to the possible mechanisms of antiviral action of plant antimicrobials.

The viruses mentioned in the cited research studies are listed in Table 16.1 along with their name abbreviations and the basic characteristics by which they may be generally categorized. This chapter has been divided into separate sections discussing enveloped and non-enveloped viruses since the mode of antiviral action, and thus the effectiveness of various plant antimicrobials, are likely to vary significantly between these two major virus types.

2.1. Efficacy of Plant Antimicrobials Against Enveloped Viruses

Numerous researchers have studied plant compounds and their antiviral effects against herpesviruses. Astani et al. (2011) investigated the antiviral efficacy of star anise essential oil (consisting of about 80 % of the phenylpropanoid, trans-anethole) as well as several other plant phenylpropanoids and sesquiterpenes (trans-anethole, eugenol, β-eudesmol, farnesol, β-caryophyllene and β-caryophyllene oxide) against herpes simplex virus type 1 (HSV-1). The antimicrobials were added to HSV-1 prior to cell infection and also after the viruses were already infecting cells. Star anise oil was the most effective, inhibiting viral infectivity by >99 %, followed by β-caryophyllene, a sesquiterpene (98 % inhibition). Trans-anethole and farnesol inhibited HSV-1 infectivity by >90 %. The other compounds reduced the cell infectivity by 60–90 %.

Koch et al. (2008) observed similar inhibitory effects of anise oil as well as oils from thyme, hyssop, ginger, chamomile, and sandalwood on herpes simplex virus type 2 (HSV-2). In another study, 12 essential oils (e.g., tea tree, cypress, juniper, tropical basil, peppermint, marjoram, eucalyptus, ravensara, lavender, lemon, rosemary, and lemongrass oils) were tested against HSV-1. A 1 % concentration of these oils completely inhibited virus infectivity but had no effect on intracellular virus. Lemongrass oil was the most effective, completely inhibiting virus infectivity at a concentration of 0.1 % (Minami et al. 2003). In a study by Armaka et al. (1999), a 1 % concentration of isoborneol, a monoterpenoid alcohol and a component of several plant essential oils, showed virucidal activity against HSV-1 (~4-$\log_{10}$ reduction in cell culture infectivity) within 30 min of exposure.

Other plant compounds including tea tree oil (Carson et al. 2001), eucalyptus oil (Schnitzler et al. 2001), Australian tea tree oil (Schnitzler et al. 2001), hyssop oil (Schnitzler et al. 2007), thyme oil (Schnitzler et al. 2007; Koch et al. 2008), manuka oil (Reichling et al. 2005), oregano oil (Siddiqui et al. 1996), ginger oil (Schnitzler et al. 2007), sandalwood oil (Schnitzler et al. 2007), carvacrol (Lai et al. 2012), thymol (Lai et al. 2012), eugenol (Lai et al. 2012), and menthol (Lai et al. 2012) have also been shown to have antiviral activity against herpesviruses. Crude extracts from *Guazuma ulmifolia* (mutumba) and *Stryphnodendron adstringens* stem bark (barbatimão) inhibited bovine herpesvirus type 1 (BHV-1) replication in infected cells (Felipe et al. 2006).

Table 16.1 Viruses referenced in this paper and their basic characteristics

Virus Name	*Abbreviation*	*External characteristic*	*Virus family*	*Viral genome*
Avian infectious bronchitis virus	AIBV	Enveloped	*Coronaviridae*	(+)ssRNA
Bovine herpesvirus type #	BHV-type #*	Enveloped	*Herpesviridae*	dsDNA
Bovine viral diarrhea virus	BVDV	Enveloped	*Flaviviridae*	(+)ssRNA
Dengue virus types 1–4	DENG-1, 2, 3, 4	Enveloped	*Flaviviridae*	(+)ssRNA
Herpes simplex virus types 1 & 2	HSV-1, HSV-2	Enveloped	*Herpesviridae*	dsDNA
Human cytomegalovirus	HCMV	Enveloped	*Herpesviridae*	dsDNA
Human immunodeficiency virus type 1	HIV-1	Enveloped	*Retroviridae*	ssRNA
Human respiratory syncytial virus	HRSV	Enveloped	*Paramyxoviridae*	(−)ssRNA
Influenza A virus	INFV-A	Enveloped	*Orthomyxoviridae*	(−)ssRNA
Influenza B virus	INFV-B	Enveloped	*Orthomyxoviridae*	(−)ssRNA
Junin virus	JUNV	Enveloped	*Arenaviridae*	(−)ssRNA
Measles virus	MeV	Enveloped	*Paramyxoviridae*	(−)ssRNA
Mumps virus	MuV	Enveloped	*Paramyxoviridae*	(−)ssRNA
Newcastle disease virus	NDV	Enveloped	*Paramyxoviridae*	(−)ssRNA
Ross River virus	RRV	Enveloped	*Togaviridae*	(+)ssRNA
SARS coronavirus	SARS-CoV	Enveloped	*Coronaviridae*	(+)ssRNA
Viral hemorrhagic septicemia virus	VHSV	Enveloped	*Rhabdoviridae*	(−)ssRNA
Yellow fever virus	YFV	Enveloped	*Flaviviridae*	(+)ssRNA
Adenovirus type #*	AdV-type #*	Non-Enveloped	*Adenoviridae*	dsDNA
Bovine rotavirus Group A	BRV-A	Non-Enveloped	*Reoviridae*	dsRNA
Coxsackie virus type #*	CV-type #*	Non-Enveloped	*Picornaviridae*	(+)ssRNA
Echovirus type #*	EV-type #*	Non-Enveloped	*Picornaviridae*	(+)ssRNA

(continued)

Table 16.1 (continued)

Virus Name	*Abbreviation*	*External characteristic*	*Virus family*	*Viral genome*
Feline calicivirus F9	FCV-F9	Non-Enveloped	*Caliciviridae*	(+)ssRNA
Fowl adenovirus	FAdV	Non-Enveloped	*Adenoviridae*	dsDNA
Hepatitis A virus	HAV	Non-Enveloped	*Picornaviridae*	(+)ssRNA
Human norovirus	HuNoV	Non-Enveloped	*Caliciviridae*	(+)ssRNA
Human rhinovirus type 2	HRV-2	Non-Enveloped	*Picornaviridae*	(+)ssRNA
Human rotavirus	RV	Non-Enveloped	*Reoviridae*	dsRNA
Murine norovirus type 1	MNV-1	Non-Enveloped	*Caliciviridae*	(+)ssRNA
Poliovirus type #	PV-type #*	Non-Enveloped	*Picornaviridae*	(+)ssRNA
Simian rotavirus SA-11	RV-SA-11	Non-Enveloped	*Reoviridae*	dsRNA
MS2 bacteriophage	MS2	Non-Enveloped	*Leviviridae*	(+)ssRNA
ΦX174 bacteriophage	ΦX174	Non-Enveloped	*Microviridae*	ssDNA
T4 bacteriophage	T4	Non-Enveloped	*Myoviridae*	dsDNA

*Abbreviation used in the current paper

#Have numerous types that are referred to in the text. The “type #” would be replaced in the actual name (or abbreviation). For instance, Coxsackie virus type B1 (CV-B1) and poliovirus type 1 (PV-1)

A potent antiviral activity against human immunodeficiency virus type 1 (HIV-1) and HSV-1 was observed inside of infected lymphocytes and macrophages *in vitro* using an aqueous extract from the South American medicinal plant *Baccharis trinervis* (Palomino et al. 2002). Garcia et al. (2010) evaluated seven essential oils from plants in Argentina against the following enveloped viruses: HSV-1, HSV-2, dengue virus type 2 (DENV-2), and junin virus (JUNV). The latter was the most resistant to essential oils. The most effective antiviral was essential oil from *Lantana grisebachii* against DENV-2, HSV-1, and HSV-2.

Ocazionez et al. (2010) found that essential oils from *Lippia alba* and *L. citriodora* were effective against DENV types 1 through 4 although oil from *L. alba* was more effective at lower concentrations than that from *L. citriodora*. These two essential oils, along with the oils from *Oreganum vulgare* and *Artemisia vulgaris*, were also shown to be effective against yellow fever virus (YFV; an enveloped virus) in a previous study (Meneses et al. 2009). *L. origanoides* oil at 11.1 µg/ml and *L. alba*, *O. vulgare* and *A. vulgaris* oils at 100 µg/ml produced 100 % reduction in the virus yield. Citral, a significant component of lemongrass oil, also exhibited antiviral activity against YFV and HSV-1 in previous studies (Astani et al. 2010; Gómez et al. 2013).

In another study (Pilau et al. 2011), Mexican oregano (*Lippia graveolens*) essential oil and its major component carvacrol were evaluated against several enveloped viruses including HSV-1 (acyclovir resistant and sensitive strains), human respiratory syncytial virus (HRSV), BHV-1, BHV-2, BHV-5, and bovine viral diarrhea virus (BVDV). BHV-1 and BHV-5 were not inhibited by the oregano essential oil, whereas the other five viruses were. Carvacrol was not effective against BHV-2. In addition, carvacrol was not as effective as oregano essential oil against other viruses in the study suggesting that some other component of oil was responsible for antiviral activity.

Yamada et al. (2009) determined that hydroxytyrosol, a small-molecule phenolic compound extracted from olive tree leaves, inactivated influenza A viruses (INFV-A) (H1N1, H3N2, H5N1, and H9N2 strains) and Newcastle disease virus (NDV) in both concentration and time-dependent manners. Olive leaf extracts have also been shown to be effective against HIV-1 (Bao et al. 2007; Lee-Huang et al. 2003) and viral hemorrhagic septicemia virus (VHSV) of fish (Micol et al. 2005). A sesquiterpene (triptofordin C-2) had moderate virucidal activity against several enveloped viruses including HSV-1, human cytomegalovirus (HCMV), measles virus (MeV), and INFV-A (H1N1 strain) (Hayashi et al. 1996). Siddiqui et al. (1996) found that oregano oil and clove oil were effective against enveloped viruses HSV-1 and NDV.

Two phytocompounds, betulinic acid and savinin, were shown to have antiviral activity against the SARS (severe acute respiratory syndrome) coronavirus (SARS-CoV) (Wen et al. 2007). Loizzo et al. (2008) studied the antiviral activity of essential oils obtained from berries and fruits of plant species in Lebanon. They found strong antiviral activity of *Laurus nobilis* oil (from berries) and

moderate antiviral activity of *Thuja orientalis* oil (from fruit) and *Juniperus oxycedrus* ssp. *oxycedrus* oil (from berries) against SARS-CoV. *Pistacia palaestina* essential oil (from fruit) was inactive against SARS-CoV.

2.2. Efficacy of Plant Antimicrobials Against Non-Enveloped Viruses

Several studies have included a comparison of the antiviral efficacy of plant antimicrobials against both enveloped and non-enveloped viruses. Not surprisingly, the observed antiviral effect has been greater for enveloped viruses in general. For instance, a sesquiterpene (triptofordin C-2) had moderate virucidal activity against several enveloped viruses (HSV-1, HCMV, MeV, and INFV-A), but was not effective against the non-enveloped poliovirus type 1 (PV-1) and Coxsackie virus type B1 (CV-B1) (Hayashi et al. 1996). Oregano oil and clove oil were effective against HSV-1 and NDV, but not against the non-enveloped PV-1 and Adenovirus type 3 (AdV-3) (Siddiqui et al. 1996). Hydroxytyrosol, a small-molecule phenolic compound extracted from olive tree leaves, inactivated INFV-A (H1N1, H3N2, H5N1, and H9N2 strains) and NDV, but was not effective against bovine rotavirus Group A (BRV-A) or fowl adenovirus (FAdV), two non-enveloped viruses (Yamada et al. 2009).

Garozzo et al. (2009) examined tea tree oil and its main antimicrobial components, terpinen-4-ol (36.7 % of total oil), γ-terpinene (22.2 %), α-terpinene (10.1 %), ρ-cymene (2.5 %), terpinolene (3.5 %), and α-terpineol (2.7 %) against HSV-1, HSV-2, and INFV-A (subtype H1N1), as well as the following non-enveloped viruses: PV-1, echovirus type 9 (EV-9), CV-B1, and adenovirus type 2 (AdV-2). Tea tree oil, terpinen-4-ol, terpinolene, and α-terpineol had an inhibitory effect on INFV-A. Only a slight antiviral effect was observed for 0.125 % (vol/vol) tea tree oil against HSV-1 and HSV-2. None of the antimicrobials was effective against any of the non-enveloped viruses. In another study (Pilau et al. 2011), Mexican oregano (*Lippia graveolens*) essential oil and its major component carvacrol were evaluated against the enveloped viruses HSV-1 (acyclovir resistant and sensitive strains), HRSV, BHV-1, BHV-2, BHV-5, and BVDV. Human rotavirus (RV; non-enveloped) was also included in the study. RV, BHV-1, and BHV-5 were not inhibited by the oregano essential oil, whereas the other five viruses were inhibited. Carvacrol alone was effective against RV but not against BHV-2.

In contrast, Semple et al. (2001) showed that chrysophanic acid (an anthraquinone) from the Australian Aboriginal medicinal plant *Dianella longifolia* inhibited poliovirus-induced cytopathic effects in BGMK (Buffalo green monkey kidney) cells for poliovirus types 2 and 3 (PV-2 and PV-3); however, it did not have significant antiviral activity against two enveloped viruses, HSV-1 and Ross River virus (RRV), or against related non-enveloped enteroviruses: Coxsackie virus types A21 and B4 (CV-A21 and CV-B4, respectively) and human rhinovirus type 2 (HRV-2).

In recent studies, non-enveloped viruses have been studied separately from enveloped viruses. For example, Búfalo et al. (2009) studied the plant *Baccharis dracunculifolia* (extract and essential oil), Brazilian propilis (the resinous substance produced by honeybees from this plant), and caffeic and cinnamic acids against PV-1. The extract worked better than the essential oil and they both worked better than the individual components (caffeic and cinnamic acids). In a study by Tait et al. (2006), homoisoflavonoids were evaluated against several enteroviruses. None were effective against PV-1 but all had marked antiviral activity against CV-B1, CV-B3, CV-B4, and CV-A9, and echovirus type 30 (EV-30).

In the last few years, there has been renewed interest in examining plant antimicrobials against viral foodborne pathogens or their surrogates. Su et al. (2010a) examined the efficacy of cranberry juice and cranberry proanthocyanidins (PAC) on enteric virus surrogates: murine norovirus type 1 (MNV-1), feline calicivirus F9 (FCV-F9), MS2 (a ssRNA bacteriophage), and ΦX174 (a ssDNA bacteriophage). MNV-1 was reduced by 2.06-$\log_{10}$ PFU/ml with cranberry juice, and 2.63-$\log_{10}$ PFU/ml with 0.15 mg/ml PAC. FCV-F9 was undetectable after exposure to both cranberry juice and 0.15 mg/ml PAC. MS2 titers were reduced by 1.14-$\log_{10}$ with cranberry juice and by 0.96-$\log_{10}$ PFU/ml with 0.60 mg/ml PAC. ΦX174 titers were reduced by 1.79-$\log_{10}$ with cranberry juice and by 4.98-$\log_{10}$ PFU/ml with 0.60 mg/ml PAC. Neutralizing the pH of the cranberry juice did not diminish the antiviral effect.

Su et al. (2011) also evaluated pomegranate juice and pomegranate phenolic extracts (PPE) against FCV-F9, MNV-1, and MS2 phage. After 20 min of exposure to pomegranate juice, reductions of 3.1- and 0.8-$\log_{10}$ of FCV-F9 and MNV-1 infectivity, respectively, were observed. A 4 mg/ml concentration of PPE resulted in slightly higher reductions of 5.1- and 1.1-$\log_{10}$ of the two viruses, respectively, following 20 min of exposure. Neither pomegranate juice nor PPE was effective against MS2 phage after 20 min.

Elizaquível et al. (2013) studied the antiviral efficacy of clove, oregano, and zataria essential oils against the human norovirus (HuNoV) surrogates, FCV-F9 and MNV-1 at 4 and 37 °C. Concentrations of 2 % oregano oil, 1 % clove oil, and 0.1 % zataria oil were less effective at 4 °C than at 37 °C. At 4 °C, MNV-1 titers were reduced by 0.6-, 0.8-, and 1.0-$\log_{10}$, respectively, while reductions of 1.6-, 0.7-, and 0.3-$\log_{10}$ were observed at 37 °C. The oils were not effective against FCV-F9 at 4 °C ($\leq$0.25-$\log_{10}$ reduction) but were highly effective at 37 °C (3.8-, 3.8-, and 4.5-$\log_{10}$ reductions for oregano, clove, and zataria oils, respectively).

Gilling et al. (2014a) examined the effects of oregano oil and its primary antimicrobial component, carvacrol, against MNV-1. Both antimicrobials caused statistically significant reductions ($P \leq 0.05$) in cell culture infectivity within 15 min of exposure (~1.0-$\log_{10}$). Despite this, the MNV-1 infectivity remained stable over time with exposure to oregano oil (1.07-$\log_{10}$ after 24 h), while carvacrol was far more effective, producing up to 3.87-$\log_{10}$ reductions

within 1 h of exposure and >4.15-$\log_{10}$ after 3 h. In a separate study, allspice oil, lemongrass oil, and citral (a component of lemongrass oil) were moderately effective against MNV-1, producing significant reductions of 0.9–1.88-$\log_{10}$) after 6 h (Gilling et al. 2014b).

The antiviral effects of grape seed extract were studied against the foodborne pathogen hepatitis A virus (HAV; non-enveloped) and the surrogates FCV-F9, MNV-1, and MS2 bacteriophage (Su and D'Souza 2011). FCV-F9 was significantly reduced (in a dose-dependent manner) by up to 4.98-$\log_{10}$ PFU/ml, MNV-1 by up to 1.97-$\log_{10}$ PFU/ml, MS2 by up to 1.85-$\log_{10}$ PFU/ml, and HAV up to 2.89-$\log_{10}$ PFU/ml after treatment at 37 °C with grape seed extract. Comparable reductions of up to 5.01-, 1.67-, 1.16-, and 3.01-$\log_{10}$ were observed for the four viruses, respectively, with grape seed extract at room temperature.

In a study by Li et al. (2012), grape seed extract was effective against non-enveloped human norovirus GII.4 (HuNoV GII.4) and MNV-1. There was a reduction in MNV-1 infectivity by >3.0-$\log_{10}$, and in HuNoV GII.4 specific binding to Caco-2 cells by >1.0-$\log_{10}$ genomic copies/ml (as determined by real time quantitative RT-PCR) following treatment. The anti-MNV-1 activity was quite limited for surface disinfection (<1-$\log_{10}$ PFU/ml after a 10-minute exposure on stainless steel discs), but a 1.5–2-$\log_{10}$ PFU/ml reduction in MNV-1 cell culture infectivity was observed with 2 mg/ml of grape seed extract used for a sanitizing wash water for fresh-cut iceberg lettuce. The grape seed extract was effective regardless of the chemical oxygen demand (0–1500 mg/ml) of the wash water.

3. MECHANISMS OF ANTIVIRAL ACTION

Due to the numerous components found in most essential oils, they often have multiple effects on the bacterial cell (Burt 2004). They may cause deterioration of the cell wall (Thoroski et al. 1989; Burt 2004), damage to cell membranes (Ultee et al. 2002) and cell membrane proteins (Ultee et al. 1999), increased membrane permeability and leakage of cell contents (Ultee et al. 2002; Burt 2004), coagulation of cytoplasm (Gustafson et al. 1998), reduction of proton motive force (Ultee et al. 1999), inactivation of critical enzymes (Wendakoon and Sakaguchi 1995; Cowan 1999; Ayala-Zavala et al. 2008), and disturbance of genetic material functionality (Ayala-Zavala et al. 2008).

The mechanisms of antiviral action of plant antimicrobials have not been studied in detail and thus are not well understood. Due to the differences between bacterial cells and virus particles (both enveloped and non-enveloped), the mechanisms are likely quite different. Nevertheless, some overlap may occur. For instance, antimicrobials that act on the outer phospholipid membrane of Gram-negative bacteria may also be active against the envelope of some viruses. In addition, antimicrobials which act on proteins or on nucleic acid may cause similar effects in both classes of microorganisms.

It is difficult to determine if reductions in virus infectivity are due to direct damage to the virus particles or to a simple inhibition of virus adsorption to the host cells. The viral inactivation might not even require a metabolic process. For instance, the virus may be immobilized to a surface, the antimicrobial could cause the virus particles to clump, the host-cell receptors may be blocked by the antimicrobial bound to virus surfaces, viral replication may be inhibited, or the nucleic acid within the viral capsid may be inactivated (Thurman and Gerba 1989). The presence or absence of a viral envelope is likely to play a significant role in the effectiveness (or lack thereof) of the antimicrobial since the exterior surface of the virus is what makes first contact with the antimicrobial.

3.1. Mechanisms of Antiviral Activity Against Enveloped Viruses

To date, the antiviral mechanisms of action for plant essential oils and other components have not been adequately evaluated. The majority of this work has been performed with enveloped viruses and with clinical treatments in mind; therefore, the focus has been on the inhibition of viral adsorption to host cells or viral uptake into the cells by plant antimicrobials or on examining their effectiveness against already intracellular viruses.

In a study of star anise essential oil and several plant phenylpropanoids and sesquiterpenes, Astani et al. (2011) found that the plant antimicrobials worked on HSV-1 directly and had no antiviral effect ($P \geq 0.2$) once the virus was inside the cell. The authors speculated that the plant compounds either: (1) inactivated the virus directly, (2) interfered with the virus envelope, or (3) blocked viral structures that are necessary for virus adsorption or entry into host cells. Koch et al. (2008) also found similar results for cell infectivity with oils from anise, thyme, hyssop, ginger, chamomile, and sandalwood with HSV-2. In addition, they found that pretreatment of the host cells with essential oils did not prevent viral infection.

Minami et al. (2003) reported that 12 essential oils (tea tree, cypress, juniper, tropical basil, peppermint, marjoram, eucalyptus, ravensara, lavender, lemon, rosemary, and lemongrass oils) completely inhibited HSV-1 cell culture infectivity but had no effect on intracellular viruses. Isoborneol, a component of several plant essential oils, caused approximately a 4-$\log_{10}$ reduction in HSV-1 cell culture infectivity and specifically inhibited the glycosylation of viral proteins but did not appear to prevent virus adsorption to the cells (Armaka et al. 1999). Likewise, the plant sesquiterpene triptofordin C-2 did not appear to prevent HSV-1 adsorption to host cells (Hayashi et al. 1996). Two phytocompounds, betulinic acid and savinin, were shown to have antiviral activity against the SARS-CoV. Both compounds exhibited protease inhibition and appeared to inhibit post-binding entry of the virus into cells (Wen et al. 2007).

Ocazionez et al. (2010) found that exposure to essential oils from *L. alba* and *L. citriodora* was effective against DENV when applied 2 h prior to cell adsorption, but no effect was observed for viruses already located within the

host cells. In another study, essential oils from *L. alba, L. origanoides*, *O. vulgare*, and *A. vulgaris* were effective against YFV; however, no reductions were observed when the cells were treated with essential oils prior to virus adsorption (Meneses et al. 2009) indicating that a different antimicrobial mechanism was operating against this virus. The essential oil from Mexican oregano (*Lippia graveolens*) inhibited HSV-1 (acyclovir resistant and sensitive strains), HRSV, and BHV-2 both before and after virus inoculation while carvacrol (its primary active component) caused inhibition only after virus inoculation (Pilau et al. 2011).

Jackwood et al. (2010) treated avian infectious bronchitis virus (AIBV) (an enveloped coronavirus) with oleoresins and essential oils from botanicals in a liquid emulsion product [QR448(a)]. The anti-AIBV activity was greater prior to virus attachment, indicating that the effect is likely directly virucidal; however, treatment of the chickens prevented symptoms but not infection for 4 days following treatment and also decreased the transmission of the virus (Jackwood et al. 2010).

In relatively few studies, the antimicrobials have been found effective against intracellular viruses. For instance, antiviral activity against HIV-1 and HSV-1 was observed inside infected lymphocytes and macrophages *in vitro* using an aqueous extract from the South American medicinal plant *Baccharis trinervis*. The effects appeared to take place primarily in the early steps of virus replication e.g., during virus-cell attachment, virus-cell fusion, and cell-to-cell fusion (Palomino et al. 2002). Garozzo et al. (2011) determined that tea tree oil and its components worked against influenza virus type A only when added within 2 h of infection. Only a slight reduction was observed when they were added during virus adsorption to the cells. The tea tree oil and its components did not interfere with virus adsorption to host cells. In addition, the treatment of infected cells with 0.01 % (vol/vol) of tea tree oil before staining seemingly inhibited viral uncoating by interfering with the acidification of the intralysosomal compartment.

Green tea extract and one of its components, epigallocatechin, was found to inhibit the acidification of endosomes and lysosomes in influenza A and influenza B infected cells in another study. The growth of these viruses was also inhibited if the cells were treated with green tea extract as early as 5 min following virus infection (Imanishi et al. 2002). Similar results were observed with the extract of *Ephedrae herba*, which inhibited the acidification of endosomes and lysosomes in cells infected with influenza A virus in a concentration-dependent manner. The growth of the virus was inhibited when cells were treated within 5–10 min following infection (Mantani et al. 1999).

Wu et al. (2010) studied the effect of On Guard™, a commercial blend of oils from wild orange, clove, cinnamon, eucalyptus, and rosemary, on H1N1 influenza virus. The treatment did not affect virus adsorption or internalization into host cells, but did result in a loss in cell culture infectivity. The treated viruses continued to express viral mRNAs but had reduced expression of viral proteins, indicating that the essential oil blend likely inhibits viral protein synthesis.

Similarly, *trans*-cinnamaldehyde inhibited influenza A viral protein but not mRNA synthesis in a previous study (Hayashi et al. 2007).

Yamada et al. (2009) determined that hydroxytyrosol, a small-molecule phenolic compound extracted from olive tree leaves, did not prevent INFV-A strain H9N2 binding to host cells, but viral mRNA and proteins were absent once the virus entered the cells. The virus also appeared to be physically altered under transmission electron microscopy (TEM), with ill-defined structure and the apparent loss of surface protein spikes. Similarly, Siddiqui et al. (1996) observed apparent dissolution of the viral envelopes of HSV-1 and NDV with both oregano and clove oil under TEM. Lai et al. (2012) also observed what appeared to be holes via TEM in the HSV-1 envelope following treatment with carvacrol and thymol. The following mechanisms appear to be the most relevant for enveloped viruses:

(1) Direct virucidal effect on the enveloped virus reducing the ability of the virus to infect host cells. Such activity does not appear to prevent virus adsorption to host cells but may include dissolution of the viral envelope or proteolytic activity that acts on virus spike proteins and receptor proteins, preventing viral entry into host cells.
(2) Inhibition of the early stages (within a few hours) of virus replication within the cells. There is apparently no (or little) effect on intracellular viruses after this time period.
(3) Inhibition of viral uncoating by reducing acidification of endosomes and lysosomes in the host cell.
(4) Inhibition of viral protein synthesis or viral protein modifications (e.g., glycosylation) inside the host cell.

It should be noted that some antimicrobials may exhibit more than one of these effects against specific enveloped viruses.

3.2. Mechanisms of Antiviral Activity Against Non-Enveloped Viruses

Recent studies have attempted to elucidate the mechanisms of action of plant antimicrobials (essential oils and extracts or their components) against non-enveloped viruses. The protein capsid in non-enveloped viruses serves to protect the viral nucleic acid and to initiate infection by facilitating virus adsorption to the host cells (Cliver 2009). In a review by Cliver (2009), it was found that antimicrobials and other treatments that inactivate small enteric viruses [caliciviruses (e.g., HuNoV, MNV-1, and FCV-F9), picornaviruses (e.g., PV-1, EV-9, CV-B1), hepatitis viruses (e.g., HAV), and astroviruses], act on the virus capsid to some extent; in many cases, the viral RNA is unaffected though the virus is no longer infectious.

Búfalo et al. (2009) studied the effects of plant *Baccharis dracunculifolia* (extract and essential oil), Brazilian propolis (the resinous substance produced by honeybees from this plant), and caffeic and cinnamic acids against PV-1. They found that treatment with the antimicrobials at the same time as

exposure to the host cells was more effective than treatment after the viruses were inside the cells. In a study by Semple et al. (2001), chrysophanic acid (an anthraquinone) from the Australian Aboriginal medicinal plant *Dianella longifolia* inhibited PV-2 and PV-3 induced cytopathic effects in BGM cells. The antimicrobial appeared to work best when added during or immediately following virus adsorption to the cells, suggesting that it acted early during the poliovirus replication cycle. In a study by Felipe et al. (2006), crude extracts from *Guazuma ulmifolia* (mutumba) and *Stryphnodendron adstringens* stem bark (barbatimão) inhibited PV-1 replication in infected cells. In another study, T4 bacteriophage was incapable of adsorbing to its bacterial host cells after exposure to cranberry juice (as visualized under scanning and transmission electron microscopy) (Lipson et al. 2007).

In a study by Cermelli et al. (2008), eucalyptus essential oil did not affect adenovirus but did cause a small reduction in cell infectivity of mumps virus (MuV). However, these researchers examined the effect of essential oil only after the viruses were inside the host cells. Based on the results for both non-enveloped and enveloped viruses in other studies, the antiviral activity of plant antimicrobials is more likely to occur prior to the internalization of the virus in the host cells. Therefore, any antiviral activity of eucalyptus oil may have been missed in this study.

Evidence of direct virucidal activity of plant antimicrobials on virus particles may be found in several published studies. Under TEM, HuNoV GII.4 virus-like particles treated with grape seed extract exhibited clumping, inflation (to approximately twice the original size), and deformation. With greater doses of grape seed extract, a large amount of protein debris was observed leading the authors to believe that this was due to direct damage to the virus capsid (Li et al. 2012). Treatment of FCV-F9 with cranberry juice and cranberry proanthocyanidins revealed structural changes in virus particles; they appeared to be damaged under TEM (Su et al. 2010b). Lipson et al. (2007) observed that rotavirus SA-11 (RV-SA11) treated with 20 % cranberry juice had single-shelled or anomalous virus-like particles, rather than the double-shelled, icosahedral "wheel-like" particles found in the untreated control samples, indicating that cranberry juice may be involved in modification of rotavirus glycoprotein spike moieties.

Although significant reductions in cell culture infectivity of MNV-1 were observed following treatment with oregano oil and carvacrol, the virus adsorption to host cells did not appear to be affected (Gilling et al. 2014a). Based on an RNase I protection assay (which indicated that the virus capsid was no longer completely intact following antimicrobial exposure), oregano oil and carvacrol appeared to exert a virucidal effect directly upon the virus capsid and, subsequently, the exposed RNA. Under TEM, the MNV-1 capsids expanded from ≤35 nm in diameter to up to 75 nm following exposure to oregano oil and up to 800 nm following exposure to carvacrol; with greater expansion, capsid disintegration could be readily observed (Gilling et al. 2014a). In a separate study, the MNV-1 capsid expanded to approximately 75 nm in

diameter following exposure to allspice oil, which also appeared to act directly upon the virus capsid (based on an RNase I protection assay) (Gilling et al. 2014b).

In studies of lemongrass oil and its major active component, citral, against MNV-1, novel mechanisms appear to play a role in their antimicrobial efficacy (Gilling et al. 2014b). Both antimicrobials appeared to coat the MNV-1 capsid, causing indiscriminate binding of the virus particles to both the host cells and the plastic of the cell culture plates. The virus capsid and the RNA genome were seemingly completely intact; however, there was a significant reduction in virus cell culture infectivity.

Based on these limited studies, the following mechanisms appear to be relevant for some non-enveloped viruses:

(1) Direct virucidal activity that reduces the ability of the non-enveloped virus to infect host cells. This would include the possible degradation of the virus capsid or protein spikes. Expansion/inflation of the virus capsid may occur which may in turn also lead to capsid disintegration. Such activity does not appear to prevent virus adsorption to host cells.
(2) Denaturation/degradation of viral nucleic acid once exposed to the antimicrobial following capsid break down.
(3) Antimicrobial coating of virus particles, leading to indiscriminate (non-specific) binding to host cells and the inhibition of cell infectivity. Capsid and viral nucleic acid are still intact.
(4) Inhibition of the early stages (within a few hours) of virus replication within the host cells. There is apparently no (or little) effect on intracellular viruses after this time period.

Some antimicrobials may exhibit more than one of these effects against specific non-enveloped viruses.

4. CONCLUSIONS

Many plant essential oils, extracts, and individual chemical components have been demonstrated to possess antiviral efficacy against enveloped and/or non-enveloped viruses. In general, plant antimicrobials exhibit greater antiviral efficacy against enveloped viruses than non-enveloped viruses (though not in all cases). There appear to be multiple mechanisms of antiviral action for plant antimicrobials; nevertheless, the majority of antimicrobials appear to act either directly on the virus itself (e.g., on the envelope or capsid) or during the early stages of virus replication following internalization of the virus into its host cell.

Although numerous studies have been conducted with enveloped viruses, few are available on non-enveloped viruses that are more likely to be the causative agents of foodborne disease. Thus, our understanding of the mechanisms

of antiviral activity against non-enveloped viruses is incomplete. Future studies should, therefore, focus on determining the effectiveness of various plant antimicrobials against enteric foodborne viruses to address such data gaps and to attempt to further elucidate the antiviral mechanisms of action.

A great challenge in employing plant essential oils/extracts/components as food sanitizers is the problem of compatibility with respect to odor and taste. Regardless of the source, phytochemicals often have specific aromatic properties and it is important, therefore, to pair such plant antimicrobials with compatible food items, much like how wine and cheese are paired. For example, green tea extract could be used with lemons, limes, apples, and other fruits traditionally combined with both hot and cold tea drinks. Grape seed extract could be used on grapes, strawberries, raspberries, and other vine-based fruits. Many essential oils are derived from food products such as garlic and oregano that are commonly used in cuisines worldwide. Additionally, spices such as cinnamon and allspice are common flavor additives used in foods. Efforts should be made to determine optimal olfactory/organoleptic combinations and to determine the lowest effective antimicrobial concentrations. This would minimize the aromatic and sensory effects and lower the costs of such food treatments.

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CHAPTER 17

Risk Assessment for Foodborne Viruses

Elizabeth Bradshaw and Lee-Ann Jaykus

1. INTRODUCTION TO RISK ANALYSIS

Risk is an inherent component of human existence, as is our creation of ways to avoid or minimize such risks. The formal process of assessing the likelihood and magnitude of risk, using that information to manage risk, and then communicating the process to others, forms the basis for risk analysis. Risk analysis pertaining to food safety is usually conducted by national, regional, and international agencies (FAO/WHO 2006), with an ultimate goal of protecting human health by producing safer food and reducing cases of human illness (FAO/WHO 2006; FAO/WHO 2003; CAC 1999). Risk analyses also help set priorities, as they can indicate where actions are most needed; which actions would be most effective at reducing risk; and where more research may be needed to fill knowledge gaps (FAO/WHO 2006). Risk analysis has classically consisted of three fundamental components: risk assessment, risk management, and risk communication (FAO/WHO 2008; CAC 1999). Knowledge generated from risk assessment is intended to drive the decisions made by risk managers and the information shared by risk communicators (CAC 1999). After a brief introduction of the latter components, this chapter will focus predominantly on risk assessment.

1.1. Risk Management

Risk management has been defined as "The process of weighing policy alternatives in the light of the results of risk assessment and, if required, selecting and implementing appropriate control options, including regulatory measures" (CAC 1999). It can be simplified to mean actions taken to mediate risks (Jaykus et al. 2006). The most important component of risk management is that the decision-making process for risk managers should be transparent

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and be based directly upon scientific findings of the risk assessment (CAC 1999; Wilde 2013). Undertaking a risk assessment before making a risk management decision is an excellent way of achieving this goal, and hence the assessment itself should be linked to a very clear risk management question. Understandably, risk managers have to balance multiple factors, such as overarching economic capabilities, the available technology, and the current social, political, and legal status of the issue at hand (EPA 2000). While risk management decisions are influenced by myriad factors, risk assessment is intended to be conducted based solely on science and hence independent of outside influences (Wilde 2013).

1.2. Risk Communication

The risk assessment process generates a significant amount of information, usually quite technical in nature. As intimated above, risk assessment is only one factor that goes into decision-making to manage risk. The third component of risk analysis, risk communication, is the sharing of risk assessment and management information among stakeholders, the general public, and other interested or affected groups (Jaykus et al. 2006), as well as among risk assessors, managers, and communicators themselves (EPA/USDA-FSIS 2012). The diversity of audience means that risk communication messages and methods must be carefully tailored to different audiences. The process may involve the creation of visual and written materials for distribution, but is more often two-way discussions with stakeholders, where the communicator appears as a credible source (Lundgren and McMakin 2013). Risk communication for human enteric pathogens is typically a planned event performed by trained professionals (FAO/WHO 2006). Aside from providing a clear explanation of the chosen risk management actions, this communication should also explain why the hazard poses a significant health risk, what populations are most at risk, what assumptions were made in the risk assessment process, where uncertainties arose in the assessment or management process, and why some actions were chosen over others (EPA/USDA-FSIS 2012; FAO/WHO 2006).

1.3. Risk Assessment

Assessment of the likelihood and magnitude of disease risk associated with a hazard is done virtually every day by most people. However, the exercise as a defined, systematic, and (frequently) quantitative entity emerged in the 1960s and 1970s in relationship to risks associated with aerospace and nuclear disasters, where their use was quite controversial. Since that time the process of risk assessment has been embraced by a number of US federal agencies, including the Occupational Safety and Health Administration, the Environmental Protection Agency (EPA), and the Departments of Agriculture, Defense, and Energy (NRC 2009; EPA 2000). For example, the US EPA has used the method from the time of its establishment in 1970. In 1983, the National Academy of Sciences (NAS) published a consensus

document entitled: *Risk Assessment in the Federal Government: Managing the Process* (NAS 1983), which has since been referred to as the "Red Book". This document established "standard operating procedures" for risk assessment and associated reporting.

Through the 1980s and early 1990s, EPA risk assessments remained focused on chemical (toxicological) agents. By the mid-1990s, there was increasing interest in applying probabilistic risk assessment principles to microbiological hazards, both in the environmental and food sectors. While the risk assessment paradigm is the same regardless of the nature of the health risk, the details differ. For example, chemicals remain stable, degrade, or perhaps change composition from the time of their deposition in the environment to human exposure, while microbes can multiply, remain stable, or even grow along the food chain. Cancer health risk modeling, which involves chronic low-level exposures, is very different from modeling the acute exposures associated with most human pathogens. Hence, in the mid-1990s, microbiological risk assessment diverged from chemical risk assessment and became its own discipline. In the last 20 years, many microbiological risk assessments have been undertaken in both environmental and food sectors.

2. MICROBIAL RISK ASSESSMENT

Microbial risk assessment (MRA) is an integral component of food and water safety management (Bartell 2010) and is considered by the Food and Agriculture Organization (FAO) of the World Health Organization (WHO) to be the method of choice for making decisions to control foodborne disease and ensure the safety of food and water supplies (FAO/WHO 2003; FAO/WHO 2006). The principal aim of MRA is to provide an objective, transparent, evidence-based assessment of the health risk of multiple exposure pathways or scenarios. The strength of MRA is illustrated in its ability to support scenario analyses, which facilitates comparison of the efficacy of different combinations of risk management interventions (Jaykus et al. 2006).

While most MRAs focus on bacterial pathogens of concern, there is an increasing interest in performing risk assessments for enteric viruses (FAO/WHO 2008). This is important because measures used to control or prevent bacterial contamination of food and water are not always effective for control of viruses (FAO/WHO 2008; CAC 2012). Microbial risk assessments for viruses have some key differences from those classically performed for chemical hazards, with added considerations such as virus decay, varying susceptibility to disinfectants, host immunity and susceptibility, differences in clinical symptoms and health outcomes (including the potential for asymptomatic infections), genetic diversity and the emergence of novel viral strains, and multiple potential routes of exposure (EPA/USDA-FSIS 2012).

3. PROCESS OF RISK ASSESSMENT

Risk assessment is a tool used to solve complex problems (Jaykus et al. 2006). It serves to fill our collective need to make decisions regarding health despite uncertainties in current knowledge (FAO/WHO 2006) and the fact that individual research findings are rarely sufficient to make management decisions (NRC 2009). The formal risk assessment process consists of four components: hazard identification, exposure assessment, hazard characterization, and risk characterization. Many of the widely-accepted definitions relating to food safety risk assessment came out of the 22nd meeting of the Codex Alimentarius Commission (CAC) in 1997 following consultations with FAO/WHO (1995), which laid significant groundwork for the international performance of risk assessment in foods.

Risk assessments can be done at many different levels for many different purposes. At the federal level, they are performed to characterize a risk either qualitatively (if there are few data) or quantitatively (if there are enough data). This can be done, for instance, to rank relative risks; prioritize risk management efforts; evaluate, on a preliminary basis, the efficacy of candidate risk mitigation strategies (through sensitivity and scenario analysis); and identify data and research needs. At the international level, risk assessments are usually used in the development and enforcement of food safety standards, particularly those involving trade (FAO/WHO 2008; CAC 1999). For example, all member nations of the World Trade Organization must partake in risk assessments in regards to their food safety and plant and animal health under Article 5 of the current Agreement on the Application of Sanitary and Phytosanitary Measures ("SPS Agreement") (WTO 1995). Risk assessments performed on behalf of international health organizations such as the FAO or WHO are generally used to inform the CAC and national governments for large-scale management of specific food-related hazards (FAO/WHO 2006).

In all cases, the risk management question(s) to be addressed by the risk assessment must be clearly delineated before the process begins lest the risk assessment veer off on to an irrelevant path. The prime directive of a risk assessment is that it should be objective, unbiased, and based in sound science (FAO/WHO 2006; NRC 2009). Ideally, in the case of foodborne pathogens, it should also take the entire food production process into account (FAO/WHO 2006), such as growing, harvesting, processing, distributing, and other actions taken before the food reaches the end consumer, also referred to as a "farm-to-fork continuum." It is not uncommon, particularly in large-scale assessments, for formal risk assessment teams to be formed. The most effective risk assessment teams are multidisciplinary, combining the expertise of epidemiologists, biostatisticians, researchers, and other specialists (FAO/WHO 2006). While risk assessments are typically portrayed as linear steps, the process should actually be seen as a series of continuous feedback loops, evolving as new information is made available. The term "iterative" is frequently used to describe this process (Jaykus et al. 2006).

4. STRUCTURE OF RISK ASSESSMENT

4.1. Hazard Identification

Hazard identification is "The identification of biological, chemical, and physical agents capable of causing adverse health effects, which may be present in a particular food or group of foods" (CAC 1999). Put simply, hazard identification involves gathering information from the body of literature to draw general conclusions about the association between a hazard and food(s), and factors impacting that association. It is generally a qualitative process with information on the hazard(s) relating to a food or practice coming from scientific literature; reports from outbreak investigations; industry-specific databases; national and international health surveillance data; consumer surveys and statistics; and consultation with experts in the field (CAC 1999).

A risk profile is a common outcome of the hazard identification step (Bouwknegt et al. 2013) and can form the basis for embarking on a full risk assessment (FAO/WHO 2006). Not every hazard and/or food merits a full risk assessment; sometimes a risk profile is sufficient to direct risk management activities (Jaykus et al. 2006; FAO/WHO 2006). Risk assessments are more likely to be undertaken when the means of exposure to a hazard is complex or poorly understood; the hazard is of significant concern to public health, regulatory, or stakeholder groups; and/or there is a need to justify whether a standing or proposed management practice is effective and has scientific merit (FAO/WHO 2006). A risk profile for a foodborne pathogen usually includes information on the current situation or status of the hazard as related to a specific product or commodity; how consumers become exposed; what factors are involved with the exposure; how the hazard might enter and affect a population, including symptoms, course of disease, and outcomes; what parts of the food production process should be evaluated in assessing risk; and how the public perceives the risks (FAO/WHO 2006; CAC 1999).

4.2. Exposure Assessment

A widely accepted definition for exposure assessment is "The qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant" (CAC 1999). An exposure assessment for food or waterborne pathogens is often an evaluation of the likelihood of the actual or anticipated intake of the pathogen in a population or certain subgroups (FAO/WHO 2003). The likelihood of exposure is determined by a chain of events which, for a full risk assessment, usually encompasses all of the steps from production to consumption (CAC 1999). Using enteric viruses in fresh produce as an example (Table 17.1), this might include considering the possibility of contamination in production waters; irrigation practices; transmission of viruses during growing or harvest; washing conditions (if applicable); persistence of the hazard on

Table 17.1 Examples of issues to address for exposure assessment associated with enteric virus risk in fresh produce

Production (growing)	*Harvesting (with packing)*	*Transport*	*Retail*	*Consumer*
Where does the contaminant originate? – Farm and field locations? – Environmental conditions? How does the contaminant reach the produce? – Focal or diffuse contamination? Contributing factors for contamination? – Water for irrigation? – Water for other purposes? – Sewage or septic tank overflows/leaks? – Defecation in fields? If contaminated, how much?	Harvesting method? – Manual vs. machine Communal equipment? Equipment sanitation? Handler/Picker hygiene and health? Field-packed, or in packing shed? Washing step? Handling of produce? Packaging setup?	Duration of travel? Temperature conditions in transit? Other environmental conditions in transit? Sanitation?	Display? – Packages or loose? Venue? – Grocery store vs. farmer's market vs. other? Temperature conditions? Degree of human handling? Misting in produce section? Shelf life of product?	Washing step? Cutting, cooking, or other preparation steps before consumption? Cross-contamination? Sanitation? Health and hygiene of consumer?

or in the produce through the food chain; and food storage, preparation, and consumption practices. An exposure assessment benefits from quantitative information about the virus of concern including its concentration in the environment and on/in foods, how often environmental contamination occurs, persistence (or growth) of the virus in the environment or foods, and portion sizes and frequency of food consumption. The end product of the exposure assessment for quantitative risk assessment is a mathematical model expressing the likelihood and magnitude of exposure to the hazard across the farm-to-fork continuum.

4.3. Hazard Characterization

Hazard characterization is "The qualitative and/or quantitative evaluation of the nature of adverse health effects associated with the hazard" (CAC 1999). Ideally, hazard characterization for an infectious agent relates to its infectivity (infectious dose); population susceptibility (genetically and/or immunologically derived); severity and endpoints of disease; and exposure dose (which is the endpoint of exposure assessment). When considering enteric viruses, especially norovirus, other factors might be important to include in hazard characterization, such as strain-specific differences in infectivity or disease severity; likelihood for evolution into new strains; modes of transmission (vomitus or fecally-derived); and propensity for secondary transmission (CAC 1999). As it is meant to be comprehensive, a hazard characterization for a pathogen may be developed in one country and used in a risk assessment performed in another country, or one performed for a waterborne pathogen may be adapted for a food-based exposure (FAO/WHO 2003).

Relating health outcomes to known levels of exposure is known as a dose–response relationship or dose–response assessment (CAC 1999). This is the ultimate product of quantitative hazard characterization and an example is shown graphically in Fig. 17.1. The dose–response curve allows for estimation of the probability of specific health impacts (e.g., infection, symptomatic illness, hospitalization, death, chronic disease) based on given exposure levels (EPA/USDA-FSIS 2012). Under ideal circumstances, dose–response data are derived from human populations. Of course, availability of direct human challenge data is scant for diseases that cause severe illness (FAO/WHO 2008). Nonetheless, some human dose–response information is available for enteric viruses such as norovirus (Teunis et al. 2008) and rotavirus (Ward et al. 1986). In the case of hepatitis A virus, the dose–response relationship is usually extrapolated from a human challenge study with echovirus 12 (Thebault et al. 2012; Schiff et al. 1984). Even with such human exposure information, only a few data points are available and hence modeling is quite crude. In the absence of human challenge data, information from epidemiology, animal feeding studies, and even laboratory-based studies on virulence or infectivity in mammalian cell culture can be used. In an ideal world, factors such as strain-

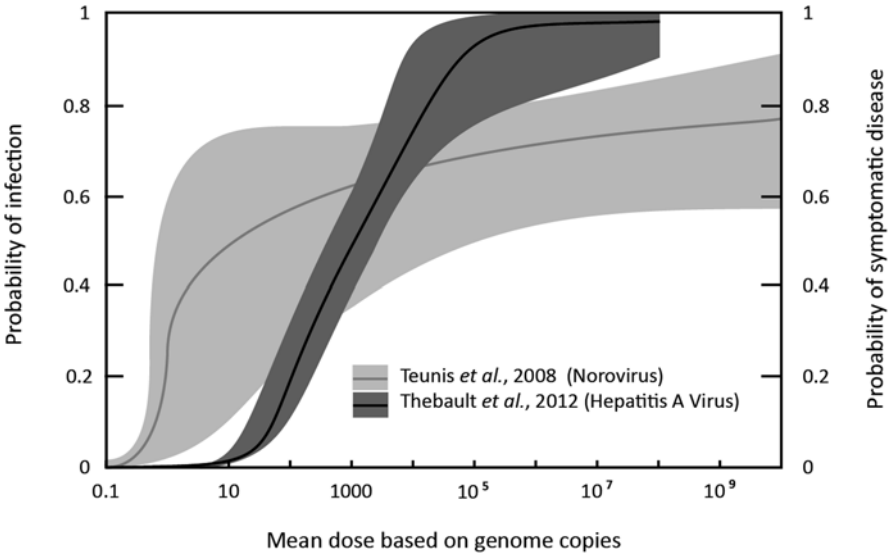

Figure 17.1. Rendering of dose–response curves with confidence intervals for Norwalk virus (without virus aggregation) and hepatitis A virus (based on a Beta-Poisson model of echovirus 12 dose–response data). Y-axis on *left* for norovirus infection, y-axis on *right* for hepatitis A symptoms. Image adapted from those presented in Teunis et al. (2008) and Thebault et al. (2012).

to-strain differences in infectivity and population susceptibility are included in dose–response modeling, but frequently data with this level of granularity are not available.

4.4. Risk Characterization

The risk characterization step combines the output of exposure assessment and hazard characterization to provide a formal evaluation of the risk (usually expressed as risk of infection or disease) from exposure to the hazard. The risk characterization process results in the formation of a risk estimate, which gives the overall likelihood and severity of the adverse effects expected in the target population (CAC 1999). As the name implies, a quantitative microbial risk assessment (QMRA) involves generating numerical values of risk for microbial agents, often using simulated models (CAC 1999; FAO/WHO 2008). In food safety, a full QMRA is usually conducted from a beginning phase in the farm-to-fork continuum, and ends with consumption of the product. However, partial QPRAM (e.g., addressing just one phase of the continuum) is also done.

Monte Carlo simulation is by far the most common approach to risk characterization modeling and is often used in exposure modeling as well (Mokhtari

and Jaykus 2009; Thebault et al. 2012). This should be apparent in several of the articles referenced later in this chapter (Mokhtari and Jaykus 2009; Thebault et al. 2012; Schijven et al. 2013; Mara et al. 2007; Mara and Sleigh 2010; Petterson et al. 2001; Bouwknegt et al. 2015). Named for a famous casino in Monaco, Monte Carlo relies on sampling random (or pseudorandom) numbers, generated like the outcomes from a roulette wheel (Zio 2013; Amar 2006), and hence is probabilistic in nature. Basically, each input or variable in the exposure or risk model is estimated as a distribution, rather than a single "point." The appropriate distribution is determined from the data source(s) or else by assumption. Then, for each risk calculation or "simulation," the input values are chosen randomly from each distribution and a single risk number is produced. This process is repeated multiple times or as multiple "iterations" (usually 10,000) (Amar 2006). Each of these is its own miniature risk assessment so to speak, and the results of all the iterations together are graphed as a distribution of probabilities and risk.

Monte Carlo simulation-based models, which rely on expressing input values as distributions, are particularly appealing because they can represent variability and uncertainty. Variability refers to data heterogeneity or variation; it is an inherent property of the data and cannot be reduced by further measurement (Suter 2006). The term variability usually applies to a well-characterized dataset. Uncertainty is basically a "lack of knowledge" or absence of data (NRC 2009; Zio 2013). While it can be reduced by further study or data collection, many input variables in a risk assessment are simply uncharacterized but still need to be included in the model. However, since their data are often assumed or at best approximated, they can lead to inaccuracy and bias in risk estimates. Remembering that simulated outcomes may not hold true in the real world, it is best to validate mathematical models with known disease incidence data or real-life information (FAO/WHO 2006). Epidemiological data are frequently used to do such validations. However, this can be difficult for viruses as there are no international requirements to report foodborne viral disease outbreaks and the vast majority are unrecognized or uninvestigated (FAO/WHO 2008).

Under the best of circumstances, risk characterization should be accompanied by sensitivity and scenario analyses (CAC 1999). In sensitivity analysis, one input variable is toggled between its low and high value keeping all the others at median, followed by simulation. This is systematically done for each input such that there is a range of low and high risk associated with each input. Sensitivity analysis identifies which variables provide the largest range of risk estimates, and the model is hence more "sensitive" to those inputs. In short, sensitivity analysis determines how changes in the entered parameters affect the results of a model (FAO/WHO 2006; CAC 1999; Zio 2013), which can be used to identify inputs having the greatest impact on the magnitude of differences in risk estimates. This can allow the user to make inferences about real-world events. Frequently, the most sensitive variables are those that also drive

the risk, and hence these are good candidates as the focus of mitigation strategies (Barker 2014).

In scenario analysis, model inputs are changed based on how the user anticipates they might be impacted by a candidate mitigation strategy. The simulations are then rerun, and the risk estimates produced are compared to those of the baseline model. In this way, the user can estimate how one or more candidate control measures might impact risk, allowing for comparative analysis and a scientific, risk-based approach to decision-making. Sensitivity analysis also enables the user to simulate an array of feasible scenarios and to estimate which single or combined management actions are likely to have the biggest impacts on risk (Zio 2013), or little impact at all. In addition, both sensitivity and scenario analyses can help to identify inputs having the greatest level of uncertainty. These are usually areas in need of additional research or data collection.

Monte Carlo simulation has proven extremely useful in QMRA because it is able to integrate complex data sets in a systematic manner, thereby incorporating variability and uncertainty in model design and risk estimates. Ultimately, this provides risk estimates that more closely mimic what might happen in real-life situations (Zio 2013; Amar 2006). Historically, Monte Carlo-based calculations have required immense programing and computing power, but technology advances have allowed widespread use of this modality (Zio 2013).

5. ELEMENTS OF RISK ASSESSMENT IN FOOD VIROLOGY

Understanding that many of the world's current food safety measures may not be effective for preventing enteric viral diseases, an expert panel met at the request of the WHO and the FAO in 2008 to discuss the importance of viruses in foods. The attendees compiled a list of foodborne viruses of primary concern on a global scale, based on criteria such as the health and economic costs of disease, incidence and prevalence, level of difficulty in their control, and impacts on trade. The final list included hepatitis A virus (HAV), human norovirus (NoV), and human rotavirus (HRV) (the three most important viruses), along with some emerging viruses of concern: hepatitis E virus (HEV), highly pathogenic avian influenza virus (HPAI-H5N1), SARS-coronavirus, and Nipah virus. The group also identified virus-food commodity pairings of concern based on the current body of knowledge. The most important were NoV and HAV in bivalve shellfish (including oysters, clams, cockles, and mussels), fresh produce, and prepared foods. Lastly, the expert panel identified three major routes of viral contamination of foods: human sewage and feces, infected food handlers, and animals (in the case of the zoonotic viruses) (FAO/WHO 2008). The working group concluded that a comprehensive QMRA of any one virus-commodity combination was not currently

possible, given the lack of quantitative data required for such assessments (FAO/WHO 2008). They did believe that lessons learned from similar consultations on bacterial agents, and the general concepts of risk assessment, were appropriate for evaluating viral foodborne disease risks if adequate supporting data were available (FAO/WHO 2008).

The lack of knowledge about foodborne viruses has been an ongoing issue for performing QMRAs (Bouwknegt et al. 2013; HPA 2004). Human NoV has been particularly challenging to assess because it cannot be propagated in cell culture, which has historically limited research investigations to molecular testing, the use of cultivable surrogate animal viruses, and human challenge studies (Atmar et al. 2008; FAO/WHO 2008; CAC 2012; HPA 2004). The use of surrogates requires making the assumption that they behave in a manner similar to human NoV, which is not necessarily the case (CAC 2012). Perhaps most importantly, it is generally recognized that the cultivable surrogates do not uniformly (or arguably, adequately) mimic human NoV resistance to disinfection (Hoelzer et al. 2013).

Although enteric viruses cannot multiply in foods, they are able to persist in the environment and are also quite resistant to most sanitizers and disinfectants used at regulated or manufacturer-recommended concentrations (FAO/WHO 2008). Hepatitis A virus, for example, can persist on fresh produce for longer than the item's shelf life (Croci et al. 2002; Sun et al. 2012). Enteric viruses also tend to be highly infectious and are shed into the environment in large quantities in vomit and stool. Norovirus has a low infectious dose (perhaps $\geq$ 18 viral particles) (Teunis et al. 2008). Millions to billions of virus particles may be shed per gram of stool, and for prolonged post-symptomatic periods (4–8 weeks) (Atmar et al. 2008). People may also be asymptomatic carriers of the virus and viral shedding can begin before the onset of clinical signs (Atmar et al. 2008. This suggests that a person may spread the virus to others before they even know they are infected. Additionally, while there are some common virus-food commodity pairings associated with disease, the wide variety of foods that can become contaminated, in addition to the many ways that foods can be produced, processed, and prepared, makes for many, many contamination scenarios. These are just a few of the intricacies of foodborne enteric virus transmission and illnesses that complicate efforts to accurately model and estimate potential risks to human health (FAO/WHO 2008).

5.1. Hazard Assessment, Risk Profiles, and Meta Analysis

In 2004, the Health Protection Agency (now part of Public Health England) conducted a feasibility study on future QMRAs related to enteric viruses. This was a large-scale project, involving the compilation and evaluation of a comprehensive body of information, the creation of a database to store the information, and an outline of the parameters needed for qualitative and quantitative risk assessments. The team evaluated transmission via person-to-person contact, bivalve shellfish, salad vegetables, and fruit. The final outcomes were suggestions for future research. In 2009, the U.S. Food and Drug

Administration (FDA) posted a risk profile for HAV infection associated with the consumption of fresh and fresh-cut produce (FDA 2009) and continues efforts in developing a comprehensive hazard identification on foodborne NoV; although at the time of this writing, no formal report or publication was available. The Dutch Food and Consumer Product Safety Authority has documented evidence relevant to the transmission of HAV in shellfish, NoV in fresh fruits and vegetables, and HEV in pork (Bouwknegt et al. 2013), while the New Zealand Food Safety Authority sponsored a risk profile on NoV in raw molluscan shellfish (ESR 2009).

More recently in 2014, the US National Advisory Committee on Microbiological Criteria for Foods (NACMCF) competed its response to questions posed by food safety regulatory bodies regarding control strategies for reducing foodborne NoV infections, which could be considered a type of risk profile (NACMCF 2014). Some recent systematic reviews and meta analyses have been produced to address questions regarding persistence, resistance, and infectivity of NoV (Hoelzer et al. 2013), illustrating that comprehensive data collection and analysis can be used to begin to answer key questions and identify future research needs.

5.2. Data for Exposure Modeling

Many types of data are needed to support exposure modeling including information on transmission dynamics (e.g., source and prevalence of contamination and virus transferability, etc.), virus behavior (persistence in foods and the environment, resistance to inactivation strategies, etc.), food consumption (e.g., which foods, how often consumed, serving sizes, etc.). While discussing all of these is beyond the scope of this work, they have constituted active areas of research in the last 5–10 years. By way of example, initial studies on the prevalence of human NoV (and sometimes HAV) contamination in molluscan shellfish in the U.S. (DePaola et al. 2010) and fresh-cut produce in Canada (Mattison et al. 2010) have been supplemented by more recent contamination prevalence studies, also focused on molluscan shellfish (Brake et al. 2014; Loutreul et al. 2014; Rodriguez-Manzano et al. 2013; Suffredini et al. 2014; Schaeffer et al. 2013) and fresh produce (berries and lettuce) (Loutreul et al. 2014; Pérez-Rodríguez et al. 2014; Maunula et al. 2013; De Keuckelaere et al. 2014). There is increasing interest in contamination of beef and pork with rotavirus and/or HEV (Jones et al. 2014; Wilhelm et al. 2014). Such studies have been facilitated by the availability of improved, and in some cases, more standardized methods for detecting viral contamination in foods.

Other areas of active research have been transferability of NoV and HAV in the food production and processing chain (Grove et al. 2015; Escudero et al. 2012; Tuladhar et al. 2013), virus environmental persistence (Mormann et al. 2015; D'Souza et al. 2006; Fallahi and Mattison 2011; Liu et al. 2009); virus resistance to sanitizers and disinfectants, (Cromeans et al. 2014; Tung et al. 2013; Park and Sobsey 2011), and virus inactivation strategies (Jean et al. 2011;

Fino and Kniel 2008; Leon et al. 2011). Further details of these are discussed elsewhere in this book.

5.3. Predictive Microbiology

While any risk model could be called predictive in nature, predictive microbiology is a particularly important tool in exposure assessment. In food microbiology, predictive modeling refers to the use of mathematical models to quantitatively predict the behavior of microbes in a given environment (Pérez-Rodríguez et al. 2014). Its use usually focuses on environmental persistence and resistance/inactivation by physical, chemical, or biological means. There are a number of instances in which predictive modeling has been applied to laboratory data on virus persistence and/or inactivation, for example feline calicivirus (FCV, a human NoV surrogate) and HAV with high hydrostatic pressure (Buckow et al. 2008; Kingsley et al. 2006); depuration kinetics of HAV and murine norovirus (MNV-1; another human NoV surrogate) in shellfish (Polo et al. 2015); heat inactivation of MNV-1, FCV, and HAV in deli meat by heat (Bozkurt et al. 2015); to name a few. These can all be helpful in exposure assessment depending upon the application.

In one particular study, Espinosa et al. (2012) determined the inactivation kinetics of poliovirus and rotavirus on lettuce and spinach using electron beam (E-beam) radiation. The authors then used these data to estimate a theoretical reduction in infection risk associated with this treatment. The experimental portion yielded D_{10} values of 1.0–1.3 kGy for rotavirus and 2.3–2.4 kGy for poliovirus. The risk model included parameters associated with serving size, initial virus contaminant concentration (ranging from 10^0 to 10^3 PFU/g), output from the kinetic inactivation model, and dose–response (Beta-Poisson for rotavirus and an exponential model for poliovirus). Reductions in infection risks varied widely (from negligible to over 10^4) as a function of risk assessment parameters. By way of example, treatments of 3 kGy with a starting virus population on lettuce of 10 PFU/g reduced the risk of poliovirus infection from consumption of contaminated lettuce from a baseline of >20 infections to 6 infections per 100 individuals. Under similar circumstances, rotavirus risk associated with consumption of contaminated spinach dropped from >30 infections to 5 per 100 persons. This paper provides an example in which laboratory-based work was combined with a relatively simple risk model to produce estimates of risk reduction as a function of a virus inactivation strategy.

In a similar study, Praveen et al. (2013) determined the inactivation kinetics of HAV and MNV-1 in oysters treated by E-beam irradiation at a dose of 5 kGy. The authors then used these data to estimate a theoretical reduction in infection risk associated with this treatment. Mean D_{10} values of 4.05 and 4.83 kGy were calculated for MNV-1 and HAV, respectively. The risk model included the same parameters as used in the previous study but adjusted for the product (oysters) and the hazard (Beta-Poisson model used for both). The model predicted that if the product were contaminated at a concentration of

10^5 infectious units per 12 raw oyster serving size, a 5 kGy treatment would result in a 12–16 % reduction in infection for both viruses. At only 10^2 infectious units per serving, NoV infection risk reduction remained relatively stable (26 %) but a 91 % reduction in HAV infection risk was predicted. This study showed that even at high E-beam doses, the viruses of greatest public health significance could not be eliminated from oysters, and at high E-beam doses, risk reduction was minimal.

5.4. Hazard Characterization

Most human data to support enteric virus dose–response relationships are old and/or scant. For example, human challenge studies for poliovirus (vaccine strain) (Katz and Plotkin 1967; Katz and Price 1967; Minor et al. 1981; Lepow et al. 1962), rotavirus (Ward et al. 1986), and echovirus 12 (Schiff et al. 1983; Schiff et al. 1984) are available, as are relevant animal challenge data for HAV (in nonhuman primates) (Purcell et al. 2002). Perhaps the first significant work, in which human challenge studies were used as the basis of dose–response modeling, is that of Haas (1983). This investigator compared three commonly used dose–response models with experimental data for poliovirus (three datasets) and echovirus 12 (one dataset). Specifically, the log-normal, simple exponential, and a Beta-distributed model were evaluated. The former two are deterministic models and the latter a stochastic model. For deterministic models, parameter values are determined at the outset and hence the model does not consider randomness or uncertainty. The output is hence "determined." Stochastic models allow for randomness in one or more inputs and must be analyzed statistically. The outcome is not a single value, but a group of values.

For three of the four virus datasets analyzed by Haas (1983), all three models provided satisfactory fit and produced ID_{50} (50 % infectious dose) values for each relevant enteric dataset that were quite similar. However, there were significant differences in model predictions at low doses, in which case the Beta and exponential models produced more conservative (higher) disease risks. This provided impetus for the rather wide use of Beta-distributed models for hazard characterization of many of the enteric viruses.

Human NoV challenge studies have been reported in the recent literature (Atmar et al. 2014; Teunis et al. 2008; Thebault et al. 2013) and several others have been completed but not yet published. Atmar et al. (2014) estimated an ID_{50} for Norwalk virus of 3.3 reverse transcription PCR units, which corresponded to between 1320 and 2800 genome equivalent copies, but the study did not include extensive mathematical modeling. The Teunis et al. (2008) work was the first to analyze human challenge study data to produce dose–response models for the prototype human NoV, the Norwalk virus. These investigators sought to determine the probability of infection in participants based on the hit theory, which considers microbial infection as a result of a chain of conditional events, i.e., (1) ingestion of one or more organisms based on an inoculum having a Poisson distribution; and (2) successful navigation of

the organism(s) through all host barriers with maintenance of infectivity. A beta-distributed probability is used to model the latter. Particle aggregation was taken into account mathematically and was fit to the experimental data. Probability of illness given infection was modeled by logit transformation of infection and aggregation distributions followed by production of maximum likelihood estimates. In susceptible individuals, the ID_{50} was about 1000 and 18 genome copies for aggregated virus and disaggregated virus, respectively. The investigators concluded that that the average probability of infection for a single infectious particle could be as high as 0.5. Probability of illness given infection was also dose-dependent, and ranged from 0.1 to 0.7 for 10^3 and 10^8 genome copies, respectively (Fig. 17.1).

Thebault et al. (2013) used outbreak data to design a human NoV dose–response model. Specifically, they used data from five oyster-associated outbreaks in which the exposed population and attack rates were characterized; the number of oysters consumed was known; and the concentration of NoV determined from oysters associated with the outbreak. They used a Beta binomial distribution to model infectivity that took into account heterogeneity of the host-pathogen relationship and non-uniform distribution of the pathogen in the food. Bayesian modeling was then done to estimate model parameters and predict probabilities. Median ID_{50} estimates were 1.6–7.1 genome copies per oyster. The median probability of infection for secretor-positive individuals (which are susceptible to NoV infection, unlike secretor-negative individuals) exposed to a single virus genome was around 0.29 (95 % CI 0.015–0.61) for GI human NoV and 0.4 (0.040–0.61) for GII. Illness probability was 0.13 (0.007–0.39) and 0.18 (0.017–0.42) for GI and GII, respectively. Statistically, there was no difference between infectivity of GI and GII human NoV. As expected, secretor-negative individuals had much lower probabilities of infection and illness. Overall, these data are in relative agreement with those of Teunis et al. (2008), confirming the exquisitely low infectious dose for this group of viruses.

Both Teunis et al. (2008) and Thebault et al. (2013) noted the issue of heterogeneity in response, which is not really considered in current dose–response modeling efforts. Such heterogeneity is mediated by a number of factors, including secretor status (innate, genetically predetermined immunity) and exposure-driven immunity. The ability of exposure to one human NoV strain to protect against infection with another, but only sometimes, also complicates modeling efforts. There are likely strain-to-strain differences in innate susceptibility and disease outcomes (disease severity) but these are also poorly characterized. In most instances, the inability to consider these factors in dose–response modeling means that current models tend to be more conservative (overestimate infection or disease risk) for normal, healthy individuals. This is probably not the case for sensitive subpopulations (young children and the elderly). Nonetheless, there are now solid human NoV dose–response estimates, which will likely be refined as data from GII.2 and GII.4 challenge studies are further analyzed.

Modeling the dose–response relationships for human rotavirus and HAV are more challenging. Probit analysis of human challenge study data for rotavirus suggested an ID_{50} of about 10 infectious units with an estimate that one infectious unit should infect about 25 % of susceptible adults (Ward et al. 1986). Espinosa et al. (2012) used the Beta-Poisson model for rotavirus, as described by Haas et al. (1999). Schiff et al. (1983, 1984) used probit analysis of human feeding study data for echovirus 12 to estimate that ingestion of 1–2 infectious units of this virus would infect 1 % of the population. Echovirus 12 is frequently used as a dose–response surrogate for HAV. Pintó et al. (2009), Praveen et al. (2013), and Thebault et al. (2013) all used a Beta-Poisson model to estimate HAV risks based on the work of Rose and Sobsey (1993) (Fig. 17.1). On the other hand, Bouwknegt et al. (2015) used the rotavirus data of Ward et al. (1986) and a modified exponential model in their HAV dose–response modeling. In some instances, HAV risks are expressed as infection, in others, as disease (usually jaundice).

6. RECENT RECENT RISK MODELING EFFORTS IN FOOD VIROLOGY

Most risk modeling efforts in food and environmental virology have focused on water, fresh produce, molluscan shellfish, and prepared foods. From a food perspective, these studies are described in greater detail below and summarized in Table 17.2.

6.1. Fresh Produce

Contact with sewage-contaminated water, or handling by infected food handlers practicing poor personal hygiene (contact during picking, packing, and/or food preparation) is thought to be the main route of viral contamination for fresh produce, though specific data on each are lacking (FAO/WHO 2008). There are also no universal or far-reaching guidelines on the types of water used for irrigation, and some areas of the world have higher risks of contamination of agricultural waters with human sewage (FAO/WHO 2008). Since fresh produce is often consumed raw, without prior treatments that could inactivate enteric viruses, prevention of contamination is usually considered the best option for a safe final product (Bouwknegt et al. 2015). In this section, we discuss risk-based studies focusing on irrigation waters and the farm-to-fork chain.

6.1.1. Irrigation with Wastewater or Recycled Water

Hamilton et al. (2006) developed a QMRA model for enteric viruses (as a group) in raw vegetables irrigated with non-disinfected, secondary-treated reclaimed water. The group chose this as a worst-case scenario: eating

Table 17.2 Summary of risk assessment research performed for foodborne and waterborne viruses in since 2000, in chronological order

Author	*Viruses studied*	*Sources evaluated*	*Methods used*	*Significant findings*
Petterson et al. (2001)	Enterovirus	Contaminated irrigation water	QMRA—Monte Carlo	Predicted enterovirus infection rates were more sensitive to the inactivation rate of viruses than to occasionally high virus concentrations
Hamilton et al. (2006)	Rotavirus, as a proxy for enteric viruses	Contaminated irrigation water	QMRA—Monte Carlo, Beta-Poisson	>1 day (14 day) lapse between irrigation and product consumption had most significant effect on annual infection risk
Teunis et al. (2008)	Norwalk virus	Characteristics of human infection	Human feeding study; dose–response	Based on human challenge, average probability of infection for a single norovirus particle approximated 0.5, with dose-dependent probability of illness ranging from 0.1 to 0.7
Mokhtari and Jaykus (2009)	Norovirus	Foodservice industry	QMRA—Monte Carlo	Gloving and handwashing compliance are the most effective methods for controlling contamination of ready-to-eat food products, when practiced simultaneously
Pintó et al. (2009)	Hepatitis A virus	Clams associated with an HAV outbreak to model consumption patterns	Molecular detection; best-fit dose–response model	Used lab-based data for dose–response assessment, and verified using epidemiological data
Espinosa et al. (2012)	Poliovirus Type 1 Rotavirus	Contaminated lettuce and spinach post-irradiation	Molecular detection; QMRA—Beta-Poisson	3 kGy treatment reduces baseline risk by less than tenfold

(continued)

Table 17.2 (continued)

Author	*Viruses studied*	*Sources evaluated*	*Methods used*	*Significant findings*
Thebault et al. (2012)	Hepatitis A virus	Oysters harvested from contaminated waters in France	QMRA—Monte Carlo	Testing measures as a risk management strategy are dependent upon the type of contamination (i.e., short-lived and homogeneous vs. chronic and heterogeneous)
Mara et al. (2007); Mara and Sleigh (2010)	Rotavirus Norovirus	Contaminated irrigation water and soil	QMRA—Monte Carlo	The WHO's 6-log reduction guideline would keep annual infections to an acceptably low level for 100 g of wastewater-irrigated lettuce consumed every other day
Barker (2014)	Norovirus	Contaminated irrigation water	QMRA	Current wastewater reuse practices unlikely to cause norovirus risks higher than annual health target
Thebault et al. (2013)	Norovirus	Contaminated oysters	Mathematical model	Bayesian analysis of epidemiological data showed mean probability of infection 0.3–0.4 and illness 0.1–0.2 for exposure to a mean of one NoV genome copy
Stals et al. (2015)	Norovirus	Preparation of deli sandwiches	Mathematical model design	Similar findings to Mokhtari and Jaykus 2009
Bouwknegt et al. (2015)	Norovirus Hepatitis A virus Adenovirus	Produce production from farm-to-fork in Europe	QMRA—Monte Carlo	Risk of virus contamination by hand contact > by irrigation water or cross-contamination

vegetables raw (cucumber, broccoli, cabbage, and lettuce) after irrigating them directly overhead with virus-contaminated water. Besides product type, simulations also included varying virus levels in effluent and various time since last irrigation. Data for enteric virus concentrations in non-disinfected secondary effluent came from comprehensive monitoring of sewage treatment plants. The variation in viral contamination (as a function of volume of water caught by the plants) was taken from previous studies using rotavirus for lettuce and cucumbers, and through field trials conducted by the authors on water retention for broccoli and cabbage. The timing variable was based on reported data on natural viral decay coefficients. Exposure was tied to consumption of the produce items and a Beta-binomial dose–response model was used based on previous work, which was considered representative of enteric viruses in general. The results were compared to a standard EPA benchmark of 10^{-4}, or one infection or fewer in every 10,000 people consuming treated water each year (EPA 1989).

Time since last irrigation was consistently significant in affecting the calculated annual risks of human infection, with estimates of 10^{-3}–10^{-1} for contaminated irrigation ending a day before harvest, down 10^{-9}–10^{-3} for the irrigation ending two weeks before harvest (Hamilton et al. 2006). Overall, the only cases that met the benchmark level of risk were those where the irrigation ceased two weeks before harvest. Based on sensitivity analyses, the most significant area of uncertainty in the models was the amount of produce the individual consumed. The results led the authors to conclude that a withholding period for the use of wastewater for irrigation prior to harvest could be used for risk mitigation. Since this set of simulations represented the worst-case scenario, alternative irrigation methods and post-harvest washing and disinfecting would likely result in further risk reduction.

Mara et al. (2007); Mara and Sleigh (2010) published two QMRA studies on risks to consumers following wastewater irrigation of crops, with an emphasis on simulating conditions in developing countries. In their first study, they characterized rotavirus infection risk associated with consumption of wastewater-irrigated lettuce. Parameters included virus numbers using *Escherichia coli* concentration as a reference; environmental persistence of rotavirus; and human consumption. The Beta-Poisson model was used for estimating the rotavirus dose–response relationship and risk was expressed as infection per person per year (pppy). A tolerable risk of 10^{-2} pppy based on modification of the WHO drinking water recommendations was chosen for this study (WHO 2004). Risks fell within a range of 10^{-4}–10^{-2} pppy when wastewater standards ranged from 10^3 to 10^5 CFU *E. coli*/100 ml, respectively. These results are consistent with the WHO standard of $<10^3$ fecal coliform/100 ml for unrestricted irrigation of salad crops and vegetables (WHO 1989), which would theoretically correspond to a risk of about 10^{-4} pppy. These findings were also in relatively good agreement when compared to epidemiological data collected from an outbreak.

In their second study, Mara and Sleigh (2010) estimated the risk of NoV infection associated with consuming wastewater-irrigated lettuce using methods and parameters similar to the ones above, with minor modifications and some different assumptions. For instance, the dose–response model was based on the work of Teunis et al. (2008) and a 1.1×10^{-3} pppy was designated as the tolerable norovirus disease risk based on loss in Disability Adjusted Life Years (DALYs), adapted from WHO wastewater guidelines (WHO 2006). Wastewater quality ranging from 10^6 to 10^8 CFU *E. coli*/100 ml resulted in median NoV infection risk of 1 pppy. A risk close to the tolerable level occurred at 10^1–10^2 CFU *E. coli*/100 ml. The investigators concluded that, if wastewater treatment and post-treatment together resulted in a 5–6 $\log_{10}$ reduction in *E. coli*, consistent with the 6-$\log_{10}$ reduction in rotavirus provided by the WHO (2006), it should be possible to achieve adequate NoV inactivation. This assumed that a hurdle type approach was used, one that relied on moderate inactivation during wastewater treatment and a high degree of inactivation during post-treatment, e.g., natural die-off and produce washing and/or disinfection.

Petterson et al. (2001, 2002) performed a "screening-level" risk assessment for the consumption of lettuce irrigated with secondary-treated sewage. They examined two primary factors: the quantity of human enteroviruses in irrigation water and the loss of viable virus particles on lettuce over time (up to 14 days post irrigation). They integrated data from the literature concerning enterovirus concentrations in secondary-treated effluent; the rate of decay of enteric viruses on the crop and during storage; the amount of water (and hence virus) that attaches to the surface of lettuce; lettuce consumption rates; and dose–response information, extrapolated from known data on rotaviruses. The levels of enterovirus in effluent were derived directly from treatment plant data. A bacteriophage provided the estimate for viral decay post-irrigation. Although initially the investigators reported risks higher than the EPA standard of 1 case per 10,000 people per year consuming finished (treated) water (EPA 1989), in a later erratum, they noted a miscalculation in the bacteriophage inactivation parameters, making the actual rate of decay more rapid. This would mean that estimates of infection would have been reduced and actually fallen below the EPA benchmark of 1 in 10,000 cases, highlighting the importance of virus decay rates in calculating risk.

Barker (2014) performed a very comprehensive QMRA to estimate NoV gastroenteritis risks associated with consuming vegetables irrigated with highly treated municipal wastewater in Melbourne, Australia. The study focused on vegetables that are typically eaten raw and irrigated with recycled water, specifically broccoli, cabbage, lettuce, and cauliflower. The author used published information on the prevalence and amount of NoV in raw sewage (based on sampling or epidemiology) in Melbourne; surveys on produce washing in Melbourne households; and even analyzed their own samples of water. Other inputs included the duration of NoV shedding; viral decay after a holding period (0, 1 days); and a reduction in virus numbers following a potential wash-

ing step, among others. A predictive model for efficacy of wastewater treatment was developed and a Beta-Poisson dose–response model was used based on the work of Teunis et al. (2008). Modeled scenarios included variations in water quality, time of year, type of vegetable, time from last irrigation, and qualities relating to consumers (i.e., washing practices, foods consumed, and body mass).

There were large differences in prevalence and concentration of human NoV as a function of estimation method (sampling vs. epidemiological) (Barker 2014). This resulted in highly variable annual disease burden estimates ranging from a low of 10^{-15} to a high of 10^{-6} DALYs/person/year. The sampling method provided much lower estimates (by 4–5 $\log_{10}$) than the 10^{-6} threshold, expressed as DALYs/person/year. The epidemiological method produced risk estimates that were occasionally up to 2 $\log_{10}$ DALYs/person/year above baseline. A third method, considered the most representative and which included an adjustment factor for NoV prevalence, produced disease burden estimates >2 $\log_{10}$ DALYs below threshold.

Lettuce carried the highest risk of all the produce types, but realistically, it also probably had the highest rate of consumption of the four items (Barker 2014). The daily probability of developing illness was most affected by the cumulative water treatment impacts on decreasing the viral load, followed by consumption rate, and the reduction in viral load when vegetables were washed. The initial parameters for estimating the concentration of NoV in both raw and treated sewage were the most important source of variability. In conclusion, the author opined that washing of vegetables and an irrigation withholding period before consumption were the most likely actions to significantly reduce the risk of NoV gastroenteritis. Their results also suggested the current water reuse procedures in Melbourne did not pose an increased risk of disease.

6.1.2. Fresh Produce Along the Farm-to-Fork Chain

In the only study of its kind, Bouwknegt et al. (2015) developed a farm-to-fork QMRA model to quantify the relative importance of potential contamination routes along the fresh produce supply chain. Raspberry and salad vegetable supply chains were modeled and risks associated with NoV, adenovirus (as a general indicator of human fecal matter), and HAV were evaluated. Conceptually, the model was broken down into production (including irrigation water and harvesters' hand modules) and processing (including hands, rinse water, and cross-contamination by conveyor belts modules). Virus inactivation was followed through each module, as appropriate. Three salad vegetable supply chains and two raspberry supply chains were modeled, each consisting of different combinations of inputs into each module. Due to lack of supporting data, the investigators did not consider certain contamination routes, including direct human fecal contamination in growing fields or

use of contaminated pesticides. Contamination during food preparation in consumer kitchens was also not considered.

Data for some model parameters, such as potential contamination points and viral contamination levels, came from the European VITAL project (Bouwknegt et al. 2015). Information on food handling practices, efficacy of virus transfer and removal in different settings, virus persistence over time, and others, were derived from the scientific literature. The hypergeometric dose–response model of Teunis et al. (2008) was used for NoV infection, and the exponential dose–response model of Haas et al. (1999) was used for HAV jaundice (disease).

Overall, the simulations showed very low risks. In fact, no pathogenic viruses were predicted to be found in the berry supply chain and risk per serving of lettuce was around 3×10^{-4} (6×10^{-6} to 5×10^{-3}) for NoV and 3×10^{-8} (7×10^{-10} to 3×10^{-6}) for HAV (Bouwknegt et al. 2015). However, a main source of uncertainly was in the dose–response models, not unusual for this type of work. The model demonstrated that hand contact led to more virus contamination of produce than irrigation water, wash water, or cross-contamination via conveyor belt. Sensitivity analysis results differed by product but, in general, the model was most sensitive to virus concentration at potential contamination points and virus removal efficiency (for rinsing steps). The investigators concluded that encouraging best practices in hand hygiene for this product sector would lead to optimized food safety outcomes.

6.2. Molluscan Shellfish

Fecal contamination of harvesting areas is the predominant route of enteric virus introduction in shellfish (FAO/WHO 2008). Bivalve mollusks, such as oysters, are known to bioaccumulate human enteric viruses in their gastrointestinal tracts, allowing them to become contaminated when grown in areas impacted by human fecal matter (Maalouf et al. 2011). In contrast, the chances of direct human contact with shellfish leading to contamination (e.g., from an infected individual shucking oysters) is considered to be relatively low (FAO/WHO 2008). One major data availability issue is that shellfish production and monitoring has historically been regulated based on the fecal indicators of water, such as levels of fecal coliforms or *E. coli* (FDA 2011). The presence or concentration of these fecal indicators does not correlate with viruses, and there are limited data on contamination prevalence in the absence of regular screening of shellfish and their waters for human enteric viruses.

Pintó et al. (2009) used a QMRA approach to estimate the levels of HAV in frozen, imported Peruvian coquina clams that were associated with actual outbreaks. Initially, they used molecular amplification methods to estimate virus numbers in the implicated product from two outbreaks. These data were then used to estimate exposure risk by mathematical modeling that included variables related to methodology (i.e., adjustments for recovery efficiency and infectivity); virus concentration reductions due to cooking; prevalence of con-

tamination; and consumption. Choice of the best-fit dose response model was done using a combination of the microbiological data, outbreak attack rates, and previously reported enteric virus models associated with human challenge studies. When a Beta-Poisson model was applied to echovirus 12 data, the researchers were able to estimate infection risks corresponding to consumption of lightly cooked clams on a per-batch basis, which matched the corresponding epidemiological data. A correlation between the prevalence of HAV cases and positive virus detection (44 % of samples positive) in clams associated with outbreaks was demonstrated, as juxtaposed to the absence of virus detection in clams randomly tested from the same batches as the outbreak-associated clams. This led the investigators to discuss the value of setting critical limits for potential viral contamination sources discharged into growing waters, and using targeted, direct, quantitative viral testing of shellfish to manage the situation when these critical limits are exceeded.

Thebault et al. (2012) performed a QMRA for HAV in shellfish harvested from contaminated production areas. Their final output was an overall annual risk of contracting symptomatic HAV among adult consumers of raw oysters in France. They generated two scenarios for mathematical modeling: one simulating a brief period of contamination (incidental) and the other for regular or prolonged contamination periods (endemic). Variables were similar to those described above for Pintó et al. (2009), including the use of a Beta-Poisson dose–response model corresponding to echovirus 12. Risk reduction was calculated as percent of baseline. Seasonal variation in oyster consumption was also considered.

Using the QMRA, Thebault et al. (2012) compared fourteen surveillance and risk management practices. These were subdivided into several major strategies: one using *E. coli* as an indicator; another using HAV testing with or without confirmation and at various frequencies; and the last being controlled purification with or without virus testing. The mitigation strategies were further subdivided based on parameters such as sensitivity of detection, confirmation of results, frequency of sampling, number of negative results before reopening for harvesting, and time to action. Direct HAV monitoring resulted in greater risk reduction than did the use of conventional bacterial indicators. In both contamination scenarios, twice monthly virus testing was an effective risk management strategy, avoiding about 40–50 % of the baseline cases. When contamination was accidental and homogeneous, waiting for three negative test results to reopen an area for harvesting was not effective in risk reduction. However, when contamination was endemic, waiting for the three negative test results was effective in preventing human cases. Any control measures that could reduce contamination by at least 2 $\log_{10}$ units (e.g., improving sanitation, harvesting from lower risk areas) resulted in the greatest risk reduction (87–88 %). This exercise is a good example of how QMRA can be used to aid in evaluation of candidate risk mitigation approaches.

6.3. RTE Foods and Food Handling

Ready-to-eat (RTE) foods, in this case defined as products that undergo extensive human handling without a terminal heating step, are the most significant vector for transmission of foodborne viral illness. In fact, food handler contact with raw and RTE foods was the most common source of foodborne outbreaks in the US from 2001 to 2008 (Hall et al. 2012), and was implicated in 70 % of NoV outbreaks linked to a contaminated food from 2009 to 2012 (Hall et al. 2014). RTE foods have also been associated with a number of high-profile outbreaks (Friedman et al. 2005; Malek et al. 2009; Becker et al. 2000). An infected individual practicing poor personal hygiene and especially, engaging in bare hand contact while preparing food, is the most likely cause of this phenomenon. However, such individuals can also contaminate utensils, preparation surfaces, restroom surfaces, and play areas, etc. Since human NoV is released and likely aerosolized during projectile vomiting, this can also serve as a source of virus to contaminate foods (de Wit et al. 2007; Patterson et al. 1997).

Mokhtari and Jaykus (2009) created a probabilistic exposure assessment that modeled the dynamics of transmission of human NoV in the retail food preparation environment. The model was conceptualized in accordance with the personal hygiene risk management triad that is based on the interrelationships among contaminant source, cross-contamination, and hygiene efficiency and compliance. Using the restroom environment as a reservoir, the dynamics of NoV transmission were modeled between employees' hands, food contact surfaces, and food products. Key model inputs included degree of fecal shedding, hand hygiene behaviors, efficacy of virus removal and/or inactivation, and transferability of virus between surfaces. The model was temporal in nature, beginning with an infected food handler failing to practice adequate personal hygiene, and following his/her movement through the restaurant environment, including food preparation, for an 8-hour shift.

From the model, the researchers determined key risk factors in food preparation that resulted in significant NoV contamination of foods, defined as >10 infectious particles per serving (Mokhtari and Jaykus 2009). Not unexpectedly, the simulations showed the highest virus levels to be on hands, followed by surfaces and gloves, implicating hands as the most important mode of transmission. Gloving and handwashing compliance were found to be the most important practices for preventing contamination of foods when an infected worker was present on-site. Sensitivity analysis revealed that the mass of feces on hands, the concentration of NoV in the stool of ill individuals, the number of bathroom visits by the employee, the level of compliance with glove use, and handwashing efficiency and compliance were the inputs having the greatest impact on risk. A novel aspect of this study was consideration of the joint effects of handwashing compliance and gloving compliance, or handwashing compliance and efficacy. Using what-if scenario analysis, the authors demonstrated that combinations of compliance, gloving, and efficacy are critical to

keeping contamination levels at <10 infectious particles per serving. In short, one intervention alone would not result in elimination of significant NoV contamination in the food if an infected food handler were present on the premises. Hence, control measures should take a multi-pronged approach.

Stals et al. (2015) produced a quantitative exposure model to simulate transmission of NoV from the hands of infected workers to deli sandwiches at a sandwich bar. In their model simulations, three employees performed their duties using shared cutlery and a shared work surface during a three-hour shift. The model structure was quite similar to that of Mokhtari and Jaykus (2009) although it was designed to accommodate NoV-contaminated lettuce as an ingredient. Many of the variables were also similar to those of Mokhtari and Jaykus (2009) and their values came from the published literature. The group also performed a two-week observational study at two deli establishments to estimate number of contact events between hands, foods and surfaces during food preparation. Four possible interventions were considered: hand disinfection with an alcohol-based sanitizer; surface disinfection (with a cloth containing disinfectant or no disinfectant); no bare hand contact (glove use); and handwashing after restroom use.

Simulations revealed that a single infected food handler readily transferred viruses to hands, surfaces, and sandwiches. On the other hand, use of contaminated lettuce in sandwich making yielded much lower numbers of virus particles on food. In a worst-case scenario in which two contamination sources (infected food handler and contaminated lettuce) were present and no intervention was used, most (96 %) of the sandwiches contained the pre-determined ID_{50} of >18 virus particles. Of the individual intervention measures considered, hand-washing following restroom use was the only one that had a substantial impact on transmission of virus to hands, surfaces, and foods, and the degree of impact very much depended on compliance. For example, at low and intermediate degrees of compliance with handwashing, the fraction of deli sandwiches containing >18 virus particles was 91 and 65 %, respectively. If high handwashing compliance was followed, no sandwiches exceeded the ID_{50}. Of course, using all four measures effectively reduced NoV concentrations on sandwiches to negligible levels (<7 virus particles).

6.4. Synthesis Comments

Some common conclusions can be made based on the risk-related modeling studies described in this chapter. The most compelling consensus conclusion comes from several models applied to virus infection or disease risks associated with the use of reclaimed water for irrigation of food crops (Hamilton et al. 2006; Mara et al. 2007; Mara and Sleigh 2010; Petterson et al. 2001, 2002; Barker 2014). Virtually all studies confirmed that there was minimal risk to human health from these practices, particularly when treated to achieve 4-$\log_{10}$ or more reduction in enteric viruses (EPA 1989). In the only production/processing risk assessment of its kind, Bouwknegt et al. (2015)

demonstrated that HAV and NoV risks associated with the consumption of berries and lettuce were minimal, and when elevated, hand contact was a much more significant source of virus contamination than was irrigation water or the produce washing process. Models applied to molluscan shellfish (Thebault et al. 2012; Pintó et al. 2009) focused on addressing questions associated with direct testing for virus contamination for harvest water classification and managing disease risk. Both studies concluded that such testing would be appropriate under certain circumstances, particularly when contamination was continuous.

The two studies that addressed retail food handling (Mokhtari and Jaykus 2009; Stals et al. 2015) concluded that hands of infected workers not practicing adequate personal hygiene is the predominant source of virus contamination of RTE foods. These studies were unique in their modeling of single and combined risk mitigation strategies, concluding that compliance with handwashing was the most effective control measure, but noting that a combined approach (e.g., including sanitation and gloving) would be necessary to reduce virus concentrations in these foods to levels associated with negligible risk.

7. CONCLUSIONS

The application of risk assessment principles to understand the dynamics of virus transmission via the food supply, estimate risk to human health, and evaluate potential mitigation strategies, is a relatively new area that evolved directly from earlier work in QMRA related to water. A small body of risk assessment work on foodborne viruses has, however, emerged in the last decade. Initially constrained by a paucity of data to support values and distributions for key inputs, studies on prevalence, persistence/resistance, transferability, and other environmental features of these viruses have been recently published. New human challenge studies using GII.2 and GII.4 strains have been completed (although not yet reported), which should improve hazard characterization. Standardized test methods, improved ability to discriminate virus infectivity status using molecular methods, and better surrogates are moving the field along as well. As national and international regulations begin to be promulgated, there will be an increasing incentive to perform QMRA as applied to viruses in foods.

Despite new data and models, there will continue to be hurdles. For example, we have no idea if virus-to-virus or strain-to-strain differences have any impact on likelihood of exposure or degree of public health risk. In addition, we now know that susceptibility to human NoV strains is, in part, genetically mediated but only a few dose–response models consider this fact. Hepatitis A and rotavirus vaccines are now widely used and likely provide life-long immunity, reducing the size of the susceptible population. Again, this is not usually con-

sidered in hazard characterization. Estimates of infectivity can be created, but until a cultivable human NoV strain is found, they will remain estimates. There are many different foods, human populations, and production/processing/preparation techniques, meaning that a "one size fits all" model is not really feasible. Consequently, risk assessments will be diverse and will continue to be subject to inclusion of poorly characterized or incomplete data, and assumptions. However, risk assessment remains a valuable means by which to integrate science in support of risk-based decision-making. In the absence of a crystal ball, is there anything better?

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