CHAPTER 4

Methods of Virus Detection in Foods

Sagar M. Goyal

1.0. INTRODUCTION

Viral contamination of food and water represents a significant threat to human health. Many different types of foods are implicated in food-borne outbreaks but shellfish (oysters, clams, mussels), cold foods, and fresh produce (fruits and vegetables) are considered to be the most important vehicles. In recent years, viral food-borne outbreaks have been traced to raspberries (Ponka et al., 1999), strawberries (Gaulin et al., 1999), well water (Beller et al., 1997), sandwiches (Daniels et al., 2000), and oysters (Kohn et al., 1995). The source of viral contamination of shellfish is fecal contamination of water in which they reside whereas produce may be contaminated through the use of contaminated irrigation or wash water, infected food handlers involved in the preparation and processing of food, and contact of produce with contaminated surfaces.

The cases of produce-associated outbreaks are on the rise because the consumption of such foods has increased due to health reasons and because produce may often be imported from areas lacking in strict hygienic measures. In addition, produce is usually eaten uncooked thereby eliminating the added safety factor provided by cooking. Produce-associated outbreaks attributed to a single food source have occurred in several countries simultaneously (Koopmans et al., 2003). In addition, food is also subject to intentional contamination with highly infectious pathogens including category A and B pathogens such as *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, Brucella spp., smallpox virus, filoviruses, arenaviruses, and alphaviruses.

Food-borne outbreaks are believed to cause an estimated 76 million illnesses, 5,000 deaths, and 325,000 hospitalizations annually in the United States (Mead et al., 1999). In many outbreaks the causative agent cannot be confirmed but they are suspected to be caused by viruses. It is generally believed that the number of viral food-borne outbreaks far exceeds the number currently being reported. One reason for the failure to confirm a viral etiology is the lack of sensitive and reliable methods for the detection of viruses in the implicated food. Svensson (2000) estimated that at least half of the viral food-borne disease outbreaks are not recognized because of inadequate sampling and detection methods.

Many viruses are associated with food-borne illnesses including norovirus (NV), hepatitis A virus (HAV), hepatitis E virus (HEV), and rotavirus (RV). Most of these viruses originate from the human gastroin-

testinal tract. Of these, caliciviruses (NVs) are the most important nonbacterial cause of food and waterborne disease outbreaks causing diarrhea, nausea and vomiting. Persons of all ages are affected and reinfections can occur because prior infection results only in a short term immune response. The problem of viral food-borne outbreaks has been neglected until recently probably because the diseases caused by such viruses are less severe and seldom fatal except in very young and elderly, pregnant women, and immunocompromised hosts (Gerba et al., 1996). However, viral food-borne outbreaks do cause high morbidity and suffering and hence should be investigated thoroughly.

Microbial monitoring is a useful tool in risk assessment of various food products. Simple, rapid, and sensitive methods for the detection of viruses in food and water can be used to help establish the cause and source of outbreaks (Jaykus, 1997) and to understand the epidemiologic features of the outbreak (Bresee et al., 2002). Existing methods to detect viruses in patients and in victims of food-borne outbreaks are relatively robust because infected individuals shed large number of viruses which can be easily detected by the current methods such as enzyme immunoassay (Fleissner et al., 1989), RT-PCR (reverse transcription-polymerase chain reaction; Anderson et al., 2001), and solid phase immune electron microscopy (SPIEM) using convalescent serum (Cunney et al., 2000; Girish et al., 2002). In addition, seroconversion (greater than fourfold antibody rise from acute to convalescent phase serum), as measured by enzyme immunoassay, can also be used for indirect evidence of viral infection (Gordon et al., 1990). Unfortunately, foods are rarely tested for viral contamination because simple and rapid methods for the detection of viruses in foods (except for shellfish) are not available (Leggitt and Jaykus, 2000; Koopmans et al., 2002).

The lack of sensitive surveillance systems to detect food contamination and the lack of available laboratory methods to concentrate and detect viruses in food has limited the ability of public health officials to identify or investigate outbreaks associated with widely distributed commodities or food products. One reason for the lack of these methods is that the number of viruses present in food is too small to be detected by methods used in clinical virology, although this low level viral contamination can still cause infection in a susceptible host. In addition, the direct detection and identification of viruses in food is difficult because of a large variety and complexity of foods, heterogeneous distribution of contaminating viruses in the food milieu, and the presence of substances in food that may inhibit or interfere with virus detection methods.

Another problem is that two of the most important food-borne viruses either do not grow in cell cultures (norovirus) or their primary isolation in cell cultures is very inefficient (hepatitis A virus). It is difficult to develop methods using these viruses thus necessitating the use of surrogates of these viruses for experimental studies. Although no validated model is available for these viruses, many investigators have utilized feline calicivirus (FCV) as a surrogate of NV (Slomka and Appleton, 1998; Gulati et al., 2001; Taku

et al., 2002; Duizer et al., 2004a, 2004b) because FCV is easily propagated and titrated in CRFK (Crandell-Reese feline kidney) cells.

The need to develop simple, rapid, and accurate methods for the concentration of viruses from large amounts of food cannot be overemphasized. An ideal method would produce a final sample that does not interfere with conventional or molecular virology techniques used for virus detection. In addition, the method should be able to concentrate viruses from many different types of foods so that it can be used in situations where the integrity and safety of food is in question and to help develop laboratory-based surveillance for the early and rapid detection of large, common-source outbreaks. These methods will also be helpful in the event of an actual or suspected act of agro-terrorism thereby maintaining the confidence of the public in public health authorities.

Considerable progress has been made in the development of sensitive methods for the detection of viruses in shellfish. The method consists basically of two steps. The first step is 'sample processing' in which viruses (or their nucleic acids) are removed and/or concentrated from shellfish tissues. The second 'detection' step uses either conventional virus isolation in cell culture or molecular techniques for the detection of viruses or their nucleic acids, respectively. Broadly speaking, there are two types of 'sample processing' methods that have been used for virus detection in shellfish; the whole virus concentration-detection method and nucleic acid extraction-detection method.

The nucleic acid extraction-detection method is relatively new. In this procedure total RNA (not whole virus) is extracted from oyster meat followed by RT-PCR (Coelho et al., 2003a). The direct RNA isolation protocol is simple because no fastidious concentration steps are involved as in the whole virus concentration-detection method. The disadvantage of this procedure is that no opportunity exists for the detection of infectious/viable virus particles. Legeay et al. (2000) described a simple procedure in which viral RNA was isolated directly from the shellfish extract by a guanidium thiocyanate-silica extraction method. Viral RNA was detected by RT-PCR followed by confirmation of the amplicons by hybridization with DIG-labeled specific probes. Using this procedure, as little as 20 PFU (plaque forming units) of HAV per g of shellfish tissues could be detected.

Goswami et al. (2002) described a method by which HAV RNA was detected in spiked samples of shellfish and cilantro. Total RNA was first isolated followed by isolation of poly(A)-containing RNA because HAV genomic RNA contains a poly(A) tail. The isolated RNA was amplified by RT-PCR and then re-amplified with internal primers to improve the quality and the quantity of amplified DNA. With this procedure, 0.15 $TCID_{50}$ of HAV could be detected in 0.62 g of tissue. In addition, this procedure was used to successfully detect naturally occurring HAV in clams involved in an outbreak of gastroenteritis.

In the whole virus procedure, on the other hand, the virus is extracted (eluted) from shellfish tissues in an alkaline buffer solution (Sobsey et al.,

1975; Sobsey et al., 1978; Seidel et al., 1983; Bouchriti and Goyal, 1992, 1993). This step is based on the fact that viruses can be adsorbed to, or eluted from, tissues and other solids by regulating the pH and ionic conditions of the suspending medium thus effectively separating viruses from solids. The viruses can then be concentrated from these extracts (eluates) by a concentration step involving acid precipitation, polyethylene glycol precipitation, or organic flocculation (Katzenelson et al., 1976; Bouchriti and Goyal, 1992; Atmar et al., 1995). During concentration, the volume of the extract (eluate) is reduced resulting in a small final sample that can be conveniently assayed for viruses by conventional or molecular methods. The most commonly used molecular diagnostic method is RT-PCR since a large majority of viruses in food happen to be RNA viruses. Ideally the final sample should not contain cytotoxic agents or PCR inhibitors when tested by cell culture inoculation or RT-PCR, respectively. A few methods that have been used for the detection of viruses in non-shellfish foods are a modification of methods used for shellfish. It is important, therefore, to review methods that have been developed for virus detection in shellfish.

2.0. METHODS FOR THE DETECTION OF VIRUSES IN SHELLFISH

Viruses are usually found at low levels in shellfish and cannot be detected by direct examination of shellfish extracts for viruses. Several methods have been developed over the last 30 years for the concentration of small amounts of viruses from large amounts of shellfish tissues. Some of these methods have been further modified to increase the efficiency of virus recovery and to reduce cytotoxic agents and PCR inhibitors in shellfish extracts. The first step involved in these procedures is extraction of virions by homogenization of shellfish tissues in a buffer solution followed by low speed centrifugation to remove solids. The eluted viruses can then be concentrated from eluates by e.g., acid precipitation, polyelectrolyte flocculation, adsorption-elution-ultrafiltration, elution-adsorption-precipitation, or elution-precipitation methods (Katzenelson et al., 1976; Bouchriti and Goyal, 1992; Atmar et al., 1995).

In a classical study, Sobsey et al. (1975) manipulated the pH and ionic conditions of shellfish homogenate to adsorb viruses to, or elute from, shellfish meat (adsorption-elution-precipitation method). The shellfish tissues were homogenized in 7 volumes of distilled water followed by the adjustment of pH and salinity to 5.0 and ≤1,500 mg NaCl/L, respectively. Under the conditions of acidic pH and low conductivity, the viruses adsorbed to oyster solids, which were collected by centrifugation. The viruses were then eluted from these solids by resuspending them in 0.05 M glycine buffer (pH 7.5, conductivity of ≥8,000 ppm NaCl). After centrifugation, the virus-containing supernatant was adjusted to pH 4.5, the precipitate collected by centrifugation, and then resuspended in a small volume of buffer. Using this method,

poliovirus from 100 g of oyster tissue was concentrated in a final volume of 15 ml with a recovery efficiency of 48%.

Over the years, many different modifications of the Sobsey method have been reported (Gerba and Goyal, 1978; Bouchriti and Goyal, 1993; Muniain-Mujika et al., 2000, 2003). In the elution-precipitation method, the initial adsorption step is eliminated (Richards et al., 1982). In some cases, an organic compound (such as beef extract powder) is incorporated during acid precipitation to provide a matrix to which viruses can be adsorbed (Vaughn et al., 1979). In yet another modification (elution-adsorption-elution), Goyal et al. (1979) eluted viruses from oyster tissues by homogenizing them in 0.05 M glycine buffer (pH 9.0). The virus containing supernatant was adjusted to pH 5.5 and conductivity of ≤1,500 ppm NaCl. After centrifugation, the supernatant was discarded and viruses were re-eluted from oyster solids by re-suspending them in glycine saline (pH 11.5). The overall virus recovery averaged 60%.

The choice of extraction and concentration methods depends on efficiency of virus recovery, ease and rapidity of the method, small final volume, and absence of interfering substances in the final sample. In their quest to increase virus recovery and eliminate PCR inhibitors, Dix and Jaykus (1998) used a protein-precipitating agent Pro-Cipitate for the concentration of NV from hard-shelled clams (*Mercenaria mercenaria*) in an adsorption-elution-precipitation scheme. Using this procedure along with RT-PCR and oligoprobe hybridization, they were able to detect as low as 450 RT-PCR amplifiable units of NV.

Traore et al. (1998) compared four methods of extraction and three methods of concentration. Mussel tissues in 60 gram amounts were spiked with astrovirus, HAV, or poliovirus and then extracted with borate buffer, glycine solution, saline beef, or saline beef-Freon. The viruses were then concentrated by precipitation with polyethylene glycol 6000 (PEG 6000) or PEG 8000 or by organic flocculation. Extraction with glycine solution and borate buffer resulted in significantly more RT-PCR-positive samples than the saline beef extraction method. Of the 20 different combinations of extraction and concentration methods tested, the borate buffer-organic flocculation, borate buffer-PEG 6000, and glycine solution-PEG 6000 were found to be the most efficient.

A modified procedure described by Mullendore et al. (2001) consisted of acid adsorption at pH 4.8, first elution with 0.05 M glycine, second elution with 0.5 M threonine, PEG-precipitation twice, chloroform-extraction twice, RNA-extraction, and a single round of RT-PCR. Using this procedure, HAV was detected at a seeding density of ≥1 plaque forming unit (PFU)/g of oyster. Kingsley and Richards (2001) developed a rapid extraction method for the detection of HAV and NV from shellfish using a pH 9.5 glycine buffer, PEG precipitation, Tri-reagent treatment, and purification of viral poly(A) RNA by using magnetic poly(dT) beads. When coupled with RT-PCR-based detection, this method could detect as low as 0.015 PFU of HAV and 22.4 RT-PCR units of NV in hard-shelled clams and oysters. Homogenization in glycine/NaCl buffer (pH 9.5) followed by PEG 8000 precipitation and DNA

extraction by proteinase K and phenol/chloroform treatment was used successfully by Karamoko et al. (2005) to detect adenoviruses in mussels (Mytilus sp.) harvested from Moroccan waters.

As is clear from the above discussion, methods for the detection of viruses in shellfish have been in use for a long time. Several studies have compared different methods and have documented the advantages and disadvantages of each. For example, Sunen et al. (2004) compared two processing procedures for the detection of HAV in clams. The first method involved acid adsorption, elution, PEG precipitation, chloroform extraction, and PEG precipitation while the second method was based on virus elution with glycine buffer (pH 10), chloroform extraction, and concentration by ultracentrifugation. Final clam concentrates were processed by RNA extraction or immunomagnetic capture of viruses (IMC) followed by RT-nested PCR. Although both methods of sample processing were effective in detecting HAV, the first method was more effective in removal of PCR inhibitors whereas the second method was simpler and faster.

3.0. DETECTION OF VIRUSES BY CONVENTIONAL VIRUS ISOLATION

Classical methods for detecting viral contamination of foods by inoculation of cell cultures are costly and time consuming. In addition, food extracts may be cytotoxic to the indicator cells and viruses commonly found in food either do not grow in cell cultures or grow very poorly. For example, Duizer et al. (2004b) made concerted efforts to grow NV in 27 different cell culture systems. Insulin, DMSO, and butyric acid were used as cell culture supplements to induce differentiation. In some cases, the cells and the NV-containing stool samples were treated with bioactive digestive additives. Even after five blind passages, no reproducible viral growth was observed. Similarly, Malik et al. (2005) evaluated 19 different cell types from 11 different animal species for the propagation of NV but were unsuccessful in propagating the virus.

Because of the above difficulties, molecular methods such as PCR and RT-PCR are commonly used for the detection of such viruses (Greiser-Wilke and Fries, 1994). However, these methods detect both infectious and non-infectious viruses and hence a sample positive by these techniques may or may not contain live infectious virus (Olsvik et al., 1994; Richards, 1999). Other limitations of these techniques include lack of sensitivity and specificity, high assay costs, and a level of technical expertise not available in most food-testing laboratories (Richards, 1999). To overcome this problem, integrated cell culture/strand-specific RT-PCR and integrated cell culture (ICC)/RT-PCR assays are available, that detect negative-strand RNA replicative intermediate, thus distinguishing infectious from non-infectious virus (Jiang et al., 2004). In these procedures, limited virus propagation occurs in cell cultures, which increases the amount of target material and

hence the sensitivity of the immunological or molecular method (Chironna et al., 2002; Bosch et al., 2004).

4.0. DETECTION OF VIRUSES BY MOLECULAR DIAGNOSTIC TECHNIQUES

The application of molecular techniques to diagnose and investigate disease outbreaks during recent years has led to a growing appreciation of the importance of these techniques (Koopmans et al., 2004). Such methods can also be used for molecular tracing of virus strains (Koopmans et al., 2002). However, the advantage of the conventional virus isolation procedure is that a live, infectious virus is detected whereas molecular procedures detect nucleic acid from both infectious and non-infectious virus particles. To determine food safety, it is important to know if the virus is capable of causing infection or not. In addition, direct isolation and purification of intact (whole) virions from foods prior to the application of nucleic acid amplification methods may remove PCR inhibitors. Many modifications of molecular procedures have been described as summarized below. More detailed description is provided in chapter 5.

4.1. PCR

Schwab et al. (2001) developed an RT-PCR-oligoprobe amplification and detection method using rTth polymerase, a heat-stable enzyme that functions as both a reverse transcriptase and DNA polymerase, in a single-tube, single-buffer, elevated temperature reaction. An internal standard NV RNA control was added to each RT-PCR to identify sample inhibition, and thermolabile uracil N-glycosylase was incorporated into the reaction to prevent PCR product carryover contamination. The amplicons were detected by ELISA using virus-specific biotinylated oligoprobes. Low levels of NV were detected in stools and bivalve mollusks following bioaccumulation. In addition, this method successfully detected NV in oysters implicated in an outbreak of NV gastroenteritis.

Many different variations of RT-PCR reaction have been described. For example, semi-nested or nested PCR has been used to increase the sensitivity of virus detection (Abad et al., 1997). Di Pinto et al. (2003) described an RT-PCR for the detection of HAV in shellfish (*Mytilus galloprovincialis*). The virus particles were first concentrated by polyethylene glycol followed by RT-PCR detection in a single step using primers specific for the VP3-VP1 region of the genome. The specificity of the PCR products was determined by hemi-nested PCR. Using this procedure, 0.6 PFU/25 g of shellfish homogenate could be detected.

Rigotto et al. (2005) compared conventional-PCR, nested-PCR (nPCR), and integrated cell culture PCR (ICC/PCR) to detect adenovirus in oysters seeded with known amounts of adenovirus serotype 5 (Ad5) and found that the nPCR was more sensitive (limit of detection: 1.2 PFU/g of tissue) than

conventional-PCR and ICC-PCR. Jothikumar et al. (2005) developed two broadly reactive one-step TaqMan RT-PCR assays for the detection of genogroup I (GI) and II (GII) of NV in fecal and shellfish samples. The sensitivity of these assays was found to be similar to that of an nPCR.

Loisy et al. (2005) developed a real-time RT-PCR based on one-step detection using single primer sets and probes for NV genogroups I and II. Using this method, they were able to detect 70 and 7 RT-PCR units of genogroup I and II norovirus strains, respectively, in artificially contaminated oysters. Burkhardt et al. (2002) compared a single, compartmentalized tube-within-a-tube (TWT) device for nPCR with conventional protocol of nPCR. The TWT device decreased the calicivirus assay detection limit 10-fold over that of conventional nPCR.

4.2. mPCR

Multiplex RT-PCR (mRT-PCR) can be used for the detection of several viruses in a single reaction tube (Rosenfield and Jaykus, 1999; Coelho et al., 2003b; Beuret, 2004). Rosenfield and Jaykus (1999) described an mRT-PCR for the simultaneous detection of human poliovirus type 1 (PV1), HAV and NV using three different sets of primers to produce three size-specific amplicons. Detection limits of ≤1 infectious unit (PV1 and HAV) or RT-PCR-amplifiable unit (NV) were achieved. Formiga-Cruz et al. (2005) developed a nested mRT-PCR for the detection of adenovirus, enterovirus and HAV in urban sewage and shellfish, which was able to detect all three viruses simultaneously when the concentration of each virus was equal to or lower than 1,000 copies per PCR reaction.

4.3. NASBA

Nucleic acid sequence-based amplification (NASBA) uses three enzymes (reverse transcriptase, RNaseH, and RNA polymerase) and is designed to detect single stranded RNA. The product of NASBA is also ssRNA which can be detected by gel electrophoresis followed by ethidium bromide staining. Jean et al. (2002) developed NASBA for HAV targeting the capsid protein gene VP2. The assay was able to detect 10^6 PFU of HAV artificially inoculated onto the surfaces of lettuce and blueberries. However, the method suffers from the low amount of food that can be processed and tested. In a later study, Jean et al. (2004) developed a multiplex format of NASBA method to simultaneously detect HAV and NV (genogroups I and II). The amplicons were detected and confirmed by agarose gel electrophoresis, electrochemiluminescence, and Northern hybridization. Using this method, they were able to detect all three viruses inoculated into two ready-to-eat foods (deli sliced turkey and lettuce) at 10^0 to 10^2 RT-PCR-detectable units in both food commodities.

4.4. PCR Inhibitors and Their Removal

Although PCR and RT-PCR assays provide rapid virus detection, their use in food samples may be hampered by the presence of PCR inhibitors. These

inhibitors are either present in the sample or are introduced during the concentration procedure (Atmar 1993; Abbaszadegan, 1993; Le Guyader et al., 1994). Naturally occurring substances such as clay, humic acid, and mussel tissues can act as PCR inhibitors (Lewis et al., 2000). In shellfish extracts, glycogen and acidic polysaccharides have been found to inhibit PCR (Schwab et al., 1998). The presence of endogenous inhibitors in sample concentrates can be detected by spiking a control reaction with a known amplifiable target and its respective primers.

Some of these problems can be resolved by using a processing method that effectively concentrates low number of viruses from large amounts of sample and in doing so gets rid of PCR inhibitors also. Often, re-extraction of the nucleic acid or ethanol precipitation and/or centrifugal ultrafiltration is sufficient to remove PCR inhibitors. Other methods that have been tried include PEG precipitation (Lewis and Metcalf, 1988; Shieh et al., 1999, 2000), freon extraction, ultrafiltration, silica gel adsorption-elution (Shieh et al., 2000), aluminum hydroxide precipitation (Farrah et al., 1978), hydroextraction (Farrah et al., 1977), membrane adsorption-elution (Goyal and Gerba, 1983), Sephadex columns, protein-precipitating agent (Jaykus et al., 1996), and beef extract flocculation (Gerba and Goyal, 1982; Traore et al., 1998). Kingsley et al. (2002) used digestive tissues of clams instead of the whole clam. In this method, they extracted virus by glycine extraction, PEG treatment, Tri-reagent treatment, and purification of poly(A) RNA with magnetic beads coupled to poly(dT) oligonucleotides. Le Guyader et al. (1994) suspended the final pellet of the concentrated sample in distilled water instead of phosphate buffered saline, thus effectively eliminating PCR inhibitors.

5.0. METHODS FOR VIRUS DETECTION ON ENVIRONMENTAL SURFACES

Food contact surfaces on which raw foods are processed often become contaminated with pathogens, which can subsequently be transferred to other foods prepared on those surfaces. Outbreaks of NV have often originated in food service establishments. Methods that can detect viral contamination on food contact surfaces should be helpful in efforts to control food-borne disease outbreaks. Taku et al. (2002) described a simple method for elution and detection of NV from stainless steel surfaces using feline calicivirus (FCV) as a model. Stainless steel surfaces were artificially contaminated with known amounts of FCV, followed by its elution in a buffer solution. Three methods of virus elution were compared. In the first method, moistened cotton swabs or pieces of positively charged filter (1MDS) were used to elute the contaminating virus. The second method consisted of flooding the contaminated surface with eluting buffer, allowing it to stay in contact for 15 min, followed by aspiration of the buffer (aspiration method). The third method, the scraping-aspiration method, was similar to the aspiration method, except that the surfaces were scraped with a cell scraper before

buffer aspiration. Maximum virus recovery (32% to 71%) was obtained with the scraping-aspiration method using 0.05 M glycine buffer at pH 6.5. Two methods (organic flocculation and filter adsorption elution) were compared to reduce the volume of the eluate recovered from larger surfaces. The organic flocculation method gave an average overall recovery of 55% compared to the filter-adsorption-elution method, which yielded an average recovery of only 8%. The newly developed method was validated for the detection of NV by artificial contamination of 929-cm^2 stainless steel sheets with NV-positive stool samples followed by RT-PCR for the detection of the recovered virus.

6.0. METHODS FOR VIRUS DETECTION IN NON-SHELLFISH FOODS

As stated earlier, one problem with food contamination is that viruses would be present in food in very small amounts even in the event of a deliberate contamination because of the large quantities of food involved. Although minimal contamination of food items may go undetected by direct detection methods, they remain hazardous to human health because of the low infectious dose of viruses. It is important, therefore, that any proposed method for virus detection in food should be capable of detecting small numbers of pathogens in large amounts/volumes of food. To do so, it is necessary to separate and concentrate viruses from food matrices followed by their detection by conventional and/or molecular methods (Sair et al., 2002). It is also important that the final concentrate should not be cytotoxic to cell cultures used in infectivity assays and be free of PCR inhibitors that may be co-extracted or co-concentrated from food. Alternately, the final sample can be treated in some manner to remove PCR inhibitors or the nucleic acid extraction method can be modified specifically to remove these inhibitors.

The development of robust, simple and sensitive methods to recover pathogens from produce (and other foods) will facilitate prevalence studies that are useful in risk assessment and for developing food safety guidelines. They can also be used to detect pathogens in "suspect" foods, will permit the identification of contaminated food, and improve our understanding of the modes of food contamination and pathogen transmission thereby assisting state and federal agricultural and health agencies to design methods for the prevention and control of pathogen contamination of foods.

Methods for the detection of viruses in non-shellfish foods are in their infancy. Daniels et al. (2000) used RT-PCR and sequence analysis for the first time to confirm the presence of viral nucleic acid in deli ham. The sequence of RT-PCR product was similar to that found in a stool specimen from an infant whose mother had prepared implicated sandwiches. Leggitt and Jaykus (2000) developed a method to extract and detect PV, HAV, and NV from lettuce and hamburger using an elution-concentration approach followed by detection with RT-PCR. Samples of lettuce or hamburger were artificially

inoculated with one of the three viruses and then processed by the sequential steps of homogenization, filtration, Freon extraction (hamburger), and PEG precipitation. To further reduce sample volume and to remove PCR inhibitors, a secondary PEG precipitation was added. Using this method, 50 g samples were reduced to a final volume of 3 to 5 ml with recovery efficiency of 10% to 70% for PV and 2% to 4% for HAV. Total RNA from the final sample was extracted in a small volume (30 to 40 microl) and subjected to RT-PCR amplification of viral RNA sequences. Viral RNA was consistently detected by RT-PCR at initial inoculum of $\geq 10^2$ PFU/50 g of food for PV and $\geq 10^3$ PFU/50 g for HAV.

Bidawid et al. (2000) used immunomagnetic beads-PCR (IM-PCR), positively-charged virosorb filters (F), or a combination of both methods (F-IM-PCR) to capture, concentrate and rapidly detect HAV in experimentally contaminated samples of lettuce and strawberries. Direct RT-PCR of the collected HAV-bead complex showed a detection limit of 0.5 PFU of the virus in 1-ml of wash solution from the produce. In the F-IM-PCR method, virus-containing washes from produce were passed through positively-charged virosorb filters and the captured virus was eluted with 10 ml volumes of 1% beef extract. Of the 62% filter-captured HAV, an average of 35% was eluted by the 1% beef extract but PCR amplification of 2 µl from this eluate failed to produce a clear positive signal. However, considering the large volumes used in F-IM-PCR, the sensitivity of detection could be much greater than that of the IM-PCR.

Schwab et al. (2000) developed a method to recover NV and HAV from food samples. The method involves washing of food samples with a guanidinium-phenol-based reagent, extraction with chloroform, and precipitation in isopropanol. Recovered viral RNA is amplified with HAV- or NV-specific primers in RT-PCR, using a viral RNA internal standard control to identify potential sample inhibition. By this method, 10 to 100 PCR units of HAV and NV seeded onto ham, turkey, and roast beef were detected. The method was applied to food samples implicated in an NV-associated outbreak at a university cafeteria. Sliced deli ham was positive for a genogroup II NV. Sequence analysis of the PCR-amplified capsid region of the genome indicated that the sequence was identical to that from virus detected in the stools of ill students. D'Souza and Jaykus (2002) used zirconium hydroxide to concentrate PV, HAV, and NV from food. Recovery of PV1 ranged from 16% to 59% with minimal loss to the supernatant. For both HAV and NV, RT-PCR amplicons of appropriate sizes were detected and confirmed in the pellet fraction with no visible amplicons from the supernatant.

Dubois et al. (2002) modified an elution-concentration method based on PEG precipitation to detect PV, HAV, and NV in fresh and frozen berries and fresh vegetables. The surface of produce was washed with a buffer containing 100 mM Tris-HCl, 50 mM glycine, 50 mM $MgCl_2$, and 3% beef extract (pH 9.5). PCR inhibitors and cytotoxic compounds were removed from viral concentrates by chloroform-butanol extraction. Viruses from 100 g of vegetal products could be recovered in volumes of 3 to 5 ml. The presence of virus

was detected by RT-PCR and cell culture inoculation. Using the latter method, 15% to 20% of PV and HAV were recovered from frozen raspberry surfaces. By RT-PCR, the recovery was estimated to be 13% for NV, 17% for HAV, and 45% to 100% for PV.

Sair et al. (2002) compared four methods of RNA extraction for optimizing the detection of viruses in food. Hamburger and lettuce samples, processed for virus concentration using a previously reported filtration-extraction-precipitation procedure, were inoculated with HAV or NV. Several RNA extraction methods (guanidinium isothiocyanate, microspin column, QIAshredder Homogenizer, and TRIzol) and primer pairs were compared for overall RNA yield (μg/ml), purity (A(260)/A(280)), and RT-PCR limits of detection. The use of TRIzol with the QIAshredder homogenizer (TRIzol/Shred) yielded the best RT-PCR detection, and the NVp110/NVp36 primer set was the most efficient for detecting NV from seeded food samples. A one-step RT-PCR protocol using the TRIzol/Shred extraction method and the NVp110/NVp36 or HAV3/HAV5 primer sets demonstrated improved sensitivity over the routinely used two-step method. Residual RT-PCR inhibitors were effectively removed as evidenced by the ability to detect viral RNA in food concentrates without prior dilution.

Recently, Kobayashi et al. (2004) used magnetic beads coated with an antibody to the baculovirus-expressed recombinant capsid proteins of the Chiba virus (rCV) to facilitate the capture of NV from food items implicated in an outbreak. Following immunomagnetic capture, NV bound to the beads was detected by RT-PCR. Two of the nine food items were positive for genogroup I NV, the nucleotide sequence of which was almost identical to those of NV strains detected in stool samples of ill patients. The immunocapture RT-PCR method seems simple and easy to perform and may be helpful in the detection of NV from outbreak-implicated foods.

Le Guyader et al. (2004) conducted a round-robin study to compare five different methods for the detection of three different viruses (PV, NV, and canine calicivirus as a surrogate of NV) in artificially contaminated lettuce. All five methods consisted of virus elution followed by concentration, and RNA extraction. The methods were compared for efficiency of virus recovery and removal of PCR inhibitors from the final samples. The first method (method A) consisted of virus elution in phosphate buffered saline (PBS) and Vertrel (1,1,1,2,3,4,4,5,5,5-decafluoropentane) and virus concentration by PEG precipitation. In the second method (method B), the viruses were eluted with 3% beef extract solution (pH 9.5) followed by ultracentrifugation to concentrate viruses. The eluent in the third method (method C) was 0.05 M glycine-NaCl buffer (pH 9.5) followed by chloroform-butanol (1:1, vol/vol) extraction and PEG precipitation. The fourth method (method D) consisted of PBS-Vertrel elution and ultracentrifugation. In the fifth method (method E), viruses were eluted with glycine buffer (pH 8.5) and concentrated by ultrafiltration. Methods C and E were found to result in a concentrate that was free of PCR inhibitors and yielded good virus recoveries (approximately 10 RT-PCR units of viruses per gram of lettuce).

We have recently developed a unified method for the concentration of feline calicivirus (FCV; a surrogate for human NV) from strawberries, lettuce, green onions, and cabbage (Goyal et al., unpublished data). In this study, produce was experimentally contaminated with known amounts of FCV followed by its elution in 7 volumes of beef extract-0.05 M glycine buffer (pH 8.5) by shaking the produce and eluent for 30 min. The volume of the eluate was reduced to 3–5 ml by precipitation with polyethylene glycol. Average virus recovery using this procedure was 70% and the final sample was not inhibitory to PCR. We believe that this method can be modified to test larger quantities of produce, other types of food, and other types of pathogens including bacteria.

7.0. COMPARISON OF METHODS

The choice of extraction/processing method will depend on per cent virus recovery and absence of cytotoxic agents and PCR inhibitors in the final concentrate (Arnal et al., 1999). It is important that a newly developed method be subjected to inter- and intralaboratory standardization and validation before recommending it for routine use (Romalde et al., 2002, 2004). In a multicenter, collaborative study to evaluate a method for the detection of NV in shellfish tissues, replicate samples of stomach and hepatopancreas of oysters or hard-shell clams were seeded with NV and then shipped to several laboratories, where viral nucleic acids were extracted followed by their detection by RT-PCR (Atmar et al., 1996). The sensitivity and specificity of the assay were 87% and 100%, respectively, when results were determined by ethidium bromide-staining of agarose gels followed by confirmation with hybridization with a digoxigenin-labeled, virus-specific probe.

Arnal et al. (1999) compared seven methods for detecting HAV in stool and shellfish samples. The protocols tested were either techniques for the recovery and purification of total RNA (RNAzol, PEG-CETAB, GTC-silica and Chelex) or techniques for isolating specifically HAV using a nucleotide probe or a monoclonal antibody. For stool samples, RNAzol, PEG-CETAB, and magnetic beads with antibody allowed efficient virus detection. For shellfish samples, three protocols (RNAzol, PEG-CETAB, and GTC-silica) allowed RNA to be extracted in 90% of cases. The authors suggested that the rapidity and low cost of RNAzol and GTC-silica made them the most suitable methods for routine diagnostic testing.

Ribao et al. (2004) compared several nucleic acid extraction and RT-PCR commercial kits for the detection of HAV from seeded mussel tissues and found that Total Quick RNA Cells & Tissues version mini (Talent) for RNA extraction and Superscript One-Step RT-PCR System (Life Technologies) for the RT-PCR reaction were the best. Di Pinto et al. (2004) compared two RT-PCR based techniques for the detection of HAV in shellfish. Both techniques involved virus extraction in glycine buffer followed by concentration of eluted virus by one or two PEG precipitation steps. RNA extraction was

done by the use of oligo (dT) cellulose to select poly (A) RNA or by another method in which total RNA is bound on silica membrane. The first approach was found to be less time-consuming and less technically demanding than the second method.

8.0. CONCLUSIONS

Methods for the detection of small amounts of viruses in large amounts of shellfish meat are available and have been used for surveillance and epidemiological studies. Such methods for the detection of viruses in non-shellfish food are not available because of the low number of viruses present in large amounts of food and because of complex and varied food matrices. To be successful, a virus detection method for foods needs to be simple, sensitive, and robust; the final sample should not contain cytotoxic agents and/or PCR inhibitors; and the method should be applicable to a large variety of food items. Because of the complexity of food matrices, it may often be necessary to use two different methods to maximize the validity of diagnosis (Rabenau et al., 2003).

9.0. REFERENCES

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