CHAPTER 6

Molecular Approaches for the Detection of Foodborne Viral Pathogens

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INTRODUCTION

The human enteric viruses are now recognized as major causes of acute nonbacterial gastroenteritis throughout the world. Those of primary epidemiological significance include hepatitis A virus (HAV) and the noroviruses, formerly known as the Norwalk-like viruses (NLVs), or the small round structured viruses (SRSVs) (reviewed in 88). Currently, the noroviruses consist of five genogroups: GI (prototype Norwalk virus); GII (prototype Snow Mountain Agent); GIII (prototype bovine enteric calicivirus); GIV (prototype Alphatron and Fort Lauderdale virus); and GV (prototype Murine norovirus) (Vinje, personal communication). The sapoviruses (previously called Sapporo-like viruses) are genetically related to the noroviruses and have occasionally caused viral gastroenteritis in humans. Both the noroviruses and the sapoviruses are members of the *Caliciviridae* family, an antigenically and genetically diverse group of gastrointestinal viruses (9, 39, 40). Other viruses that can cause food and waterborne disease include the adenoviruses, astroviruses, the human enteroviruses (polioviruses, echoviruses, groups A and B coxsackieviruses), hepatitis E virus,

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parvoviruses, and other relatively uncharacterized small round viruses. The rotaviruses, which are the leading cause of infantile diarrhea worldwide, are transmitted primarily by contaminated water but can on occasion be foodborne.

The human enteric viruses replicate in the intestines of infected human hosts and are excreted in the feces. Their primary mode of transmission is the fecal–oral route through contact with human fecal matter, although they may also be shed in vomitus. These viruses are readily spread by person-to-person contact, which is frequently responsible for the propagation of primary foodborne outbreaks. Contamination of foods may occur directly, through poor personal hygiene practices of infected food handlers, or indirectly via contact with fecally contaminated waters or soils (49, 50). Since viruses must survive the pH extremes and enzymes present in the human gastrointestinal tract, they are regarded as highly environmentally stable, allowing virtually any food to serve as a vehicle for their transmission (50). Although enteric viruses are unable to replicate in contaminated foods, they are able to withstand a wide variety of food processing and storage conditions. When present in contaminated food, their numbers are usually quite low, but since their infectious doses are also low, any level of contamination may pose a public health threat.

In spite of their initial recognition decades ago, the human enteric viruses can be considered "emerging" agents of foodborne disease, mainly because only recently have scientists been able to reliably detect these pathogens. In fact, prior to the advent of molecular biological techniques, epidemiological criteria were the best means, by which cases of enteric viral illness were recognized. Over the last 10 years, significant advances in nucleic acid amplification methods have made detection of enteric viruses in human clinical samples, specifically feces, all but routine.

The opposite is the case for the detection of viruses in foods. Historically, this has been done by infectivity assay using susceptible, live laboratory hosts. Host systems employed were mainly mammalian cell cultures of primate origin. Unfortunately, the epidemiologically important human enteric viruses, including the noroviruses and wild-type hepatitis A virus, cannot be propagated in mammalian cell culture systems, and so these are not viable detection options (49, 50). In the absence of *in vitro* virus propagation methods, nucleic acid amplification has been a promising alternative. In this chapter, we will discuss existing technologies that can be applied to the detection of viruses in foods, and we will address new developments and research needs for the application of these methods on a routine basis.

GENERAL DETECTION CONSIDERATIONS AND THE CHALLENGES

The development of effective virus detection methods from food commodities poses several challenges. Like many bacterial pathogens, these agents are typically present at low levels in contaminated foods. However, unlike bacterial pathogens, viruses cannot replicate in foods, making the use of traditional

food microbiological techniques of cultural enrichment and selective plating inapplicable *per se*. Therefore, the first goal in developing virus detection methods for foods is to separate and concentrate the agents from the food matrix. It is also necessary to sample relatively large volumes to assure adequate sample representation, thereby optimizing detection assay sensitivity.

VIRUS CONCENTRATION

Sample preparation prior to detection is of key importance when applying molecular methods to detect viral contamination in foods. In this regard, specific challenges include high sample volumes in relation to small amplification volumes, low levels of contamination, and the presence of residual food components that can later compromise detection (31, 37, 86, 97).

The main goals of virus concentration methods are to decrease sample volume and eliminate matrix-associated interfering substances, while simultaneously recovering most of the viruses present in the food sample. In order to achieve these goals, sample manipulations are undertaken that capitalize on the behavior of viruses to act as proteins in solutions, and to remain infectious at extremes of pH or in the presence of organic solvents. Because of their frequent association with viral foodborne disease outbreaks, early work on virus concentration and purification from foods focused mainly on bivalve molluscan shellfish. Recent research endeavors have included a broader range of at-risk foods.

Two major approaches to virus concentration, particularly as applied to shellfish commodities, are termed extraction–concentration and adsorption–elution–concentration (49). Both methods utilize conditions that promote the separation of viruses from shellfish tissues, through the use of filtration, centrifugation, adsorption, elution, solvent extraction, precipitation, and/or organic flocculation. The procedure generally begins with sample blending in a buffer, usually containing amino acids and an elevated pH. A common example of elution buffer is 0.1 M glycine-0.14 N saline, pH 9.0. A crude filtration step through a mesh material such as cheesecloth may be done to remove large sample particulates. Viruses do not sediment unaided, even at centrifugation speeds approaching 10,000 × g. Therefore, centrifugation can be used to sediment large food particles, with the recovery of the virus-containing supernatant. The next step usually involves pH manipulation or the addition of precipitation agents, creating conditions such that viruses adsorb to the remaining shellfish tissues. Upon subsequent centrifugation, the adsorbed viruses sediment with the tissues and the supernatant is discarded. Elution, whereby the viruses are desorbed from the tissues by further pH and/or ionic manipulations is then carried out. On subsequent centrifugation, the precipitated tissue is discarded, again retaining the supernatant. Using sequential steps of adsorption, elution, filtration, precipitation, and centrifugation, viruses are concentrated to small sample volumes and simultaneously purified, with the removal of large proportions of the food matrix and matrix-associated organic materials that may later compromise detection of the viruses.

Precipitation of viruses can be achieved by lowering pH, so called acid precipitation, or by the addition of polyethylene glycol (PEG). Both methods capitalize on the property that viruses behave as proteins in solution. The viruses, along with some of the matrix-associated proteins, will precipitate when the pH is lowered to that approximating the virus isoelectric point. Polyethylene glycol causes removal of water, allowing proteins to fall out of solution. Another method similar to precipitation is organic flocculation. Flocculating agents interact with organic material in the matrix, causing the formation of a gelatinous "floc," to which the viruses absorb (49, 50). In the case of acid and PEG precipitation, and in organic flocculation, the virus-containing solid materials can be readily harvested by centrifugation, usually done at fairly low speeds (e.g., $<5000 \times g$).

Further removal of matrix-associated organic materials can be done using a variety of agents. Since viruses remain infectious even after exposure these organic solvents such as chloroform, trichloro trifluoroethane (Freon) and more environmentally friendly solvents such as Vertrel (Dupont), these chemicals can be used to remove polar food components such as lipids. Alternative commercial virus purification agents such as ProCipitate and Viraffinity (LigoChem, Inc., Fairfield, NJ 07004) (31, 51, 66) are known to eliminate polysaccharides, an important matrix-associated inhibitor in shellfish and produce. The cationic detergent cetyltrimethylammonium bromide (CTAB) (6, 7, 52) also aids in the removal of polysaccharides. The use of Sephadex (23), cellulose (110), or Chelex (98) is helpful in the elimination of salts and small proteins. Ultrafiltration, a method that is frequently applied at the latter stages of a virus concentration scheme, provides reduction in volume while simultaneously purifying the sample.

VIRUS CONCENTRATION METHODS FOR SHELLFISH

The early molecular work aimed at detecting viruses in the food matrix focused almost exclusively on shellfish. The most common method utilizes some sort of virion concentration step prior to release or extraction of nucleic acid, followed by amplification. A second, less commonly used method resorts to direct nucleic acid extraction of a previously untreated food matrix, which circumvents the need for virus concentration.

The objective of the virion concentration approach is to concentrate viruses and remove inhibitors prior to nucleic acid amplification, with or without prior nucleic acid extraction. In early work by Atmar et al. (6, 7), investigators processed artificially contaminated shellfish samples using an initial concentration and utilized a purification scheme that consisted of solvent extraction and PEG precipitation steps. This was followed by nucleic acid extraction and subsequent amplification. Cetyltrimethylammonium bromide (CTAB) was added to remove residual inhibitors from crude nucleic acid extracts, and the resulting eluant was amplified using reverse transcriptase-polymerase chain reaction (RT-PCR) (6, 7). Dissecting the oysters, discarding the muscle tissue and processing only the digestive diverticula improved the PCR's detection

limits. This sampling approach is now the method of choice (11, 65, 67, 69, 90, 92). Figure 6.1 illustrates a representative sample-preparation protocol for shellfish. Note that second generation protocols frequently employ sequential PEG precipitation in addition to adsorption, elution, and solvent extraction steps (8, 16, 19, 20, 25, 26, 30, 59, 60, 63, 64).

Some investigators apply an antibody capture step to further concentrate and purify viruses from shellfish extracts prior to detection using RT-PCR.

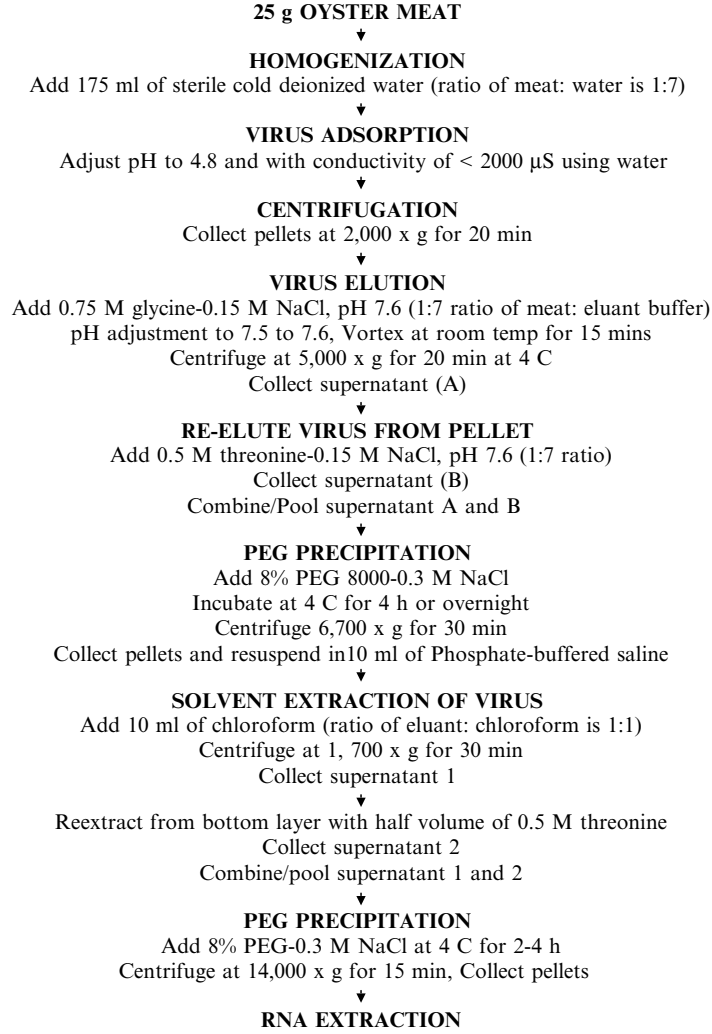

Figure 6.1. Concentration of viruses from oysters using virus adsorption, glycine-saline buffer and threonine-saline extraction, PEG precipitation, chloroform extraction, and PEG concentration as modified by Shieh et al. (95).

Desenclos et al. (29) were the first to implicate hepatitis A virus in oyster outbreak specimens by immunocapture of the virus, heat release of viral nucleic acids, and subsequent RT-PCR detection. Capitalizing on the work of Jansen et al. (48), other investigators have coated paramagnetic beads with anti-HAV IgG and used these to capture HAV from oyster extracts initially processed for virus concentration using a combination of elution, polyelectrolyte flocculation, filtration and/or ultrafiltration (28, 71). Sunen et al. (100) and Schwab et al. (94) also used antibody capture as a final virus concentration step. More recently, Kobayashi et al. (61) used magnetic beads coated with the antibody to the baculovirus-expressed recombinant capsid proteins of the Chiba virus (rCV) to capture noroviruses from food items implicated in an outbreak of acute gastroenteritis in Aichi Prefecture, Japan, detecting the virus in these foods by RT-PCR. Abd El Galil et al. (1) have used immunomagnetic separation for the detection of hepatitis A virus from environmental samples using real-time nucleic acid amplification methods.

The alternative approach of direct nucleic acid extraction and RT-PCR applied to unprocessed food sample, involves extraction of total RNA from the sample without any prior sample manipulations. This method is best suited for simple sample matrices such as the surfaces of fresh fruits and vegetables. However, Legeay et al. (65) recently reported a method that involved enzymatic liquefaction of shellfish digestive tissues, followed by clarification using dichloromethane extraction. In this case, the investigator reported that the sample could be directly processed for nucleic acid isolation and subsequent virus detection by RT-PCR.

VIRUS CONCENTRATION METHODS FOR FOODS OTHER THAN SHELLFISH

Gouvea et al. (36) first reported a systematic method for the detection of norovirus and rotavirus from representative food commodities other than shellfish, including orange juice, milk, lettuce, and melon. The method involved blending or washing, clarification by centrifugation, and removal of inhibitors by Freon extraction followed by RNA extraction. Leggitt and Jaykus (66) developed a prototype method for the concentration of poliovirus, hepatitis A virus, and Norwalk virus from 50 g samples of artificially contaminated hamburger and lettuce. The steps used included homogenization, filtration through cheesecloth, Freon extraction (hamburger only) and two sequential PEG precipitations. The sequential precipitations, which used increasing PEG concentrations, resulted in a 10- to 20-fold sample volume reduction from 50 g to approximately 2.5 ml (66). The resuspended PEG precipitate could be assayed for virus recovery by mammalian cell culture infectivity assay, when applicable, which allows for direct comparison between virus infectivity and molecular detection (104). Subsequent nucleic acid extraction resulted in an additional 100-fold sample volume reduction with detection at initial inoculum levels of $\geq 10^2$ infectious units per 50-g food sample (66). A schematic overview of this procedure is

provided in Figure 6.2. Schwab et al. (91) used TRIzol, a proprietary RNA extraction method, as a surface wash for deli meats, including samples artificially contaminated with norovirus and ones implicated in an outbreak of norovirus gastroenteritis. Although simple, the main drawback of the TRIzol surface wash method was that nucleic acid amplification inhibition persisted unless sample concentrates were diluted 10- to 100-fold. A flow diagram of this protocol is depicted in Figure 6.3.

Bidawid et al. (12) reported an immunocapture method for the concentration of hepatitis A virus from lettuce and strawberries. After surface washing to elute the viruses, the wash solution was passed through a positively charged filter, eluted, and concentrated by immunocapture. As few as 10 PFU of cell culture-adapted hepatitis A virus per piece of lettuce or strawberry could be detected by RT-PCR using this sample preparation method. However, this method too had problems with residual matrix-associated amplification inhibition.

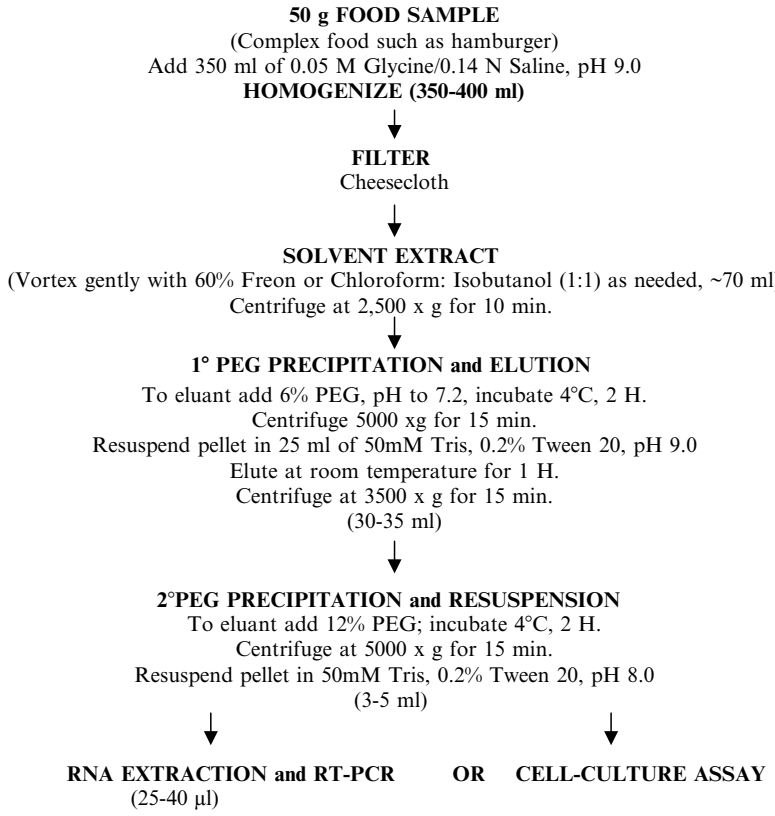

Figure 6.2. Virus concentration from hamburger/complex foods using glycine-saline buffer, chloroform extraction and two PEG extractions as followed by Leggitt and Jaykus (66)

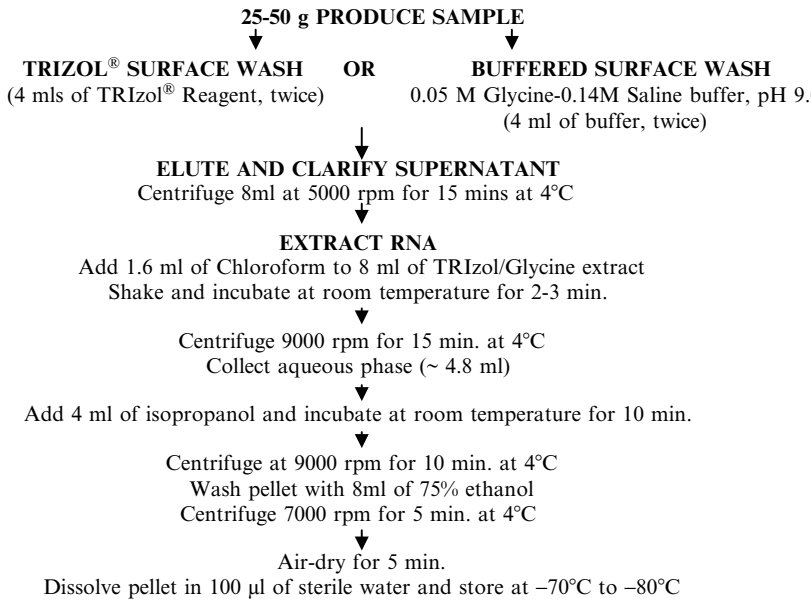

Figure 6.3. Virus concentration and extraction from produce using TRIzol® or glycine-saline buffer (modifications of protocol by Schwab et al. (91)).

As a general rule, virus concentration methods result in sample volume reductions ranging from 10- to 1000-fold. This means that a 25-g sample theoretically can be reduced to 25 µl–2.5 µl volumes with recovery of infectious virus (50). The yields after virus concentration in a food matrix have ranged from as low as 1–2% to as high as 90%. Recovery efficiency is almost always virus-specific, usually hepatitis A virus recovery is quite low when compared to recovery of other viruses such as poliovirus (reviewed in 50, 88).

Although virus concentration and sample purification steps prior to nucleic acid amplification can achieve significant sample volume reduction with relatively efficient virus recovery, there are some additional considerations when selecting a virus concentration approach. The antibody capture methods are often simpler than others as they may require fewer sample manipulations. There is also speculation that antigen-associated viral nucleic acid is more highly associated with infectious virus. However, these methods may be limited by reagent availability and high specificity, which means that only a single virus type is detected in a single assay. Other concentration and purification methods that rely on steps such as PEG precipitation and solvent extraction usually require manipulations, which may result in substantial virus loss during extraction. The direct nucleic acid extraction methods almost always result in residual RT-PCR inhibitors and often times do not provide adequate sample volume reduction.

In short, there are many virus extraction and concentration approaches that have been applied in a variety of instances. All of these methods have limitations that ultimately hinder the ability to detect the relatively low levels of virus that might be anticipated in naturally contaminated foods.

NUCLEIC ACID EXTRACTION

The earliest and simplest protocol for amplifying nucleic acids from shellfish concentrates was direct heat release followed by RT-PCR, in which case, an RNA extraction step was not applied (31, 51, 52). It soon became apparent, however, that in many instances the additional volume reductions and sample clean up provided by RNA extraction was critical to improving detection limits and circumventing the effects of residual matrix-associated amplification inhibition.

Before applying nucleic acid amplification, an efficient nucleic acid extraction step is critical in most instances. This is important because the amplification efficiency is dependent on both the purity of the target template and the quantity of target molecules obtained from the sample. Accordingly, the main goals of nucleic acid extraction are: (1) to extract and purify the nucleic acids, (2) to provide additional sample concentration, and (3) to remove residual matrix-associated inhibitory substances that could remain after the initial concentration steps are completed. The components that can hinder molecular amplification are diverse and include compounds such as divalent cations, matrix-associated components such as proteoglycans (42), polysaccharides, (6, 7, 27), glycogen, (6, 7, 51, 52), and lipids (84, 87, 91), among others. In many cases, inhibitory compounds have been largely uncharacterized. Matrix-associated inhibitors usually act by degrading the target and/or primer nucleic acids, and/or inactivating or inhibiting enzymes (86, 109, 111). Unfortunately, these inhibitory substances are frequently coextracted during virus concentration protocols, adding to the challenge of identifying reliable nucleic acid extraction processes.

Early studies used SDS-proteinase K digestion to release nucleic acids from shellfish concentrates, followed by phenol chloroform extraction with, or without the addition of cetyltrimethylammonium bromide (CTAB) to remove residual inhibitors (6, 7, 16, 90). In the last decade or so, guanidinium thiocyanate (GuSCN)-based methods became the RNA extraction method of choice largely because they are effective at deproteinization of nucleic acids while providing ample protection of RNA against native RNases. Many commercial guanidinium based kits have been used in more recent studies (4, 12, 22, 24, 25, 32, 59, 60, 75, 87, 90, 91).

Combinations of multiple extraction methods can also be used to purify nucleic acids. A simple and rapid protocol for the purification of nucleic acid that utilizes a combination of the chaotropic agent GuSCN and silica particles was first described by Boom et al. (13), and later used by others (41, 53). Other methods include a GuSCN method followed by RNA binding to glass powder, instead of silica, to provide further nucleic acid purification (63, 64).

Several investigators have compared various RNA extraction approaches specifically aimed at preparing samples for the detection of human enteric viruses. Hale et al. (44) compared four different RNA extraction methods for RT-PCR detection of noroviruses in fecal specimens, finding that the GuSCN/silica (13) method was the best at removal of inhibitors. Likewise, another study compared seven RNA extraction methods to purify hepatitis A virus RNA from stool and shellfish concentrates for RT-PCR detection (4); again, the GuSCN-silica methods were found to be the most suitable from the standpoint of speed, ease, and cost. Gouvea et al. (36) used deproteinization with GuSCN followed by adsorption of RNA to hydroxyapatite and sequential precipitation with CTAB and ethanol to purify RNA from shellfish and other selected foods. Sair et al. (87) compared multiple RNA extraction methods after concentration of noroviruses from model food commodities (hamburger sandwiches and lettuce). These included GuSCN, commercial microspin columns, the QIAshredder Homogenizer and TRIzol alone and in their various combinations. These investigators found that the use of TRIzol followed by further sample preparation using the QIAshredder Homogenizer yielded the best detection limits (<1 RT-PCR amplifiable units/reaction) for Norwalk virus preconcentrated from food samples. Similar studies by Svensson (101) demonstrated that the use of the metal chelating agent Chelex-100, or alternatively, Sephadex G200 column chromatography, during RNA extraction, provided the best RT-PCR detection limits. Others have had success using phenol-chloroform-based methods followed by further selection for viral RNA using magnetic poly (dT) beads (30, 35, 59, 60).

Despite all the efforts in identification of efficacious RNA extraction protocols, food-related amplification inhibitors frequently remain. Multiple sample manipulation steps can result in incomplete recovery and/or degradation of RNA during the extraction procedure, the consequence of which is less than optimal RNA yields (49, 50). A major problem with RNA extraction is the necessity to destroy virion integrity, thereby losing the ability to directly correlate infectivity to RT-PCR detection limits, at least when effective cell culture-based methods are available. The two main areas of active research in RNA purification are increasing yield and improving the purity of the resultant product for detection by PCR.

DETECTION

The progress in clinical detection of pathogens has always been ahead of detection in foods and many of our food methods rely on protocols initially developed for clinical samples. However, whether clinical, food, or environmental sample, the sensitivity and the specificity of molecular amplification methods is largely dependent on the choice of primers.

The genetic diversity in the *Caliciviridae* family makes primer design for the detection of the noroviruses quite challenging. Initial studies used extremely specific primers such as NV 5′/3′ and NV 36/35, which were based on sequences in

the prototype Norwalk virus genome (23, 74). With the availability of sequence information from related viruses, more broadly reactive primers have been designed. (2, 38, 57, 108). Most of the primer sequences reported are based on the highly conserved RNA-dependent RNA polymerase region of the noroviruses; occasionally the capsid region has been targeted (39, 40, 62, 81, 105).

Initially, the primer sets developed by Ando et al. (2) for the genogroup I (GI) and genogroup II (GII) Noroviruses were used as the "gold-standard" for the detection of noroviruses in clinical (fecal) and food samples (47, 80, 112). Later on, degenerate primers, or a mixture of oligonucleotide primers that vary in nucleotide sequence but have the same number of nucleotides, were developed and used for the detection of the noroviruses (38, 68, 69). The use of degenerate primers is advantageous in that all combinations of nucleic acid sequence that code for the amino acid are used in the PCR amplification. Combinations of the Ando et al. (2) GI and GII primers and various degenerate sets are routinely used in norovirus epidemiological investigations as applied to the detection of virus in fecal samples.

As with RNA extraction methods, investigators have compared the performance of various primers for the detection of a broad range of noroviruses, largely in fecal specimens. For instance, the NV110/NV36 primer set was the found to be the most efficient of the nine primer sets tested in a comprehensive study, even though it could not detect 100% of the norovirus strains tested (46). When five laboratories in five countries evaluated different RT-PCR methods on a panel of 91 fecal samples (106), no single assay was superior based on the criteria of sensitivity, detection limit, assay format, and successful implementation. However, the Boom extraction method and the use of the JV12/JV13 primer set were recommended for norovirus diagnostics.

Nonetheless, there is some lack of consensus regarding the optimal primer pair(s) to detect the noroviruses and different laboratories tend to use various methods that were developed, or are optimally suited for their purposes (112). Primers used for the detection of hepatitis A virus usually target the VP1/2A junction sequence. In this case, the issue of diversity is not relevant as it is for the noroviruses (48).

RT-PCR DETECTION OF VIRUSES IN FOODS

The choice of primers is even more critical when attempting to detect viral contamination in foods. This is because the levels of contamination are typically much lower in foods when compared to clinical samples, and even with optimal concentration and nucleic acid extraction methods, residual inhibitors often persist. Furthermore, the matrix can be responsible for nonspecific amplification and false positive results. The primers selected for the detection of viral nucleic acids derived from the food matrix should therefore have the following criteria: (1) a reasonably high annealing temperature, (2) relative nondegeneracy, and (3) broad reactivity. High stringency and primer specificity (hence the relative absence of degeneracy) are necessary to prevent nonspecific

amplification. For the genetically diverse norovirus group, the use of primers that are broadly reactive and can detect as many genetically distinct strains as possible in a single assay is essential.

The various primers used in the RT-PCR detection of noroviruses from different food matrices are summarized in Table 6.1. As with primers used in the clinical realm, these sequences correspond to mainly the viral RNA dependent RNA polymerase or the capsid protein genome regions. Primer sets targeting both the RNA polymerase and capsid genes, Mon381/383 and SR33/46, respectively, were used by Shieh et al. (96, 97) to identify a GII norovirus in oyster samples implicated in a California outbreak. For the detection of the GII noroviruses in shellfish, the NI/E3 primer set has also been used (33, 37, 63). Dubois et al. (32) used a newer primer pair to detect both noroviruses and sapoviruses in artificially contaminated produce. In a systematic comparison of four primer pairs, as applied to the detection of noroviruses in hamburger sandwiches and lettuce, Sair et al. (87) found the best detection limits using the NVp110/NVp36 primer combination. Honma et al. (46) reported that this same primer pair was broadly reactive for a range of noroviruses, eliminating the need for separate amplifications for the two norovirus genogroups (GI and GII). These primers have also been used together or in combination with NI or NVp69 for the detection of norovirus contamination in shellfish (59, 67, 69).

"Nested" RT-PCR or double amplification has been used for the detection of noroviruses (36, 63, 91, 99) and hepatitis A virus (18, 19, 20, 43, 59) in foods. This approach can improve assay sensitivity and also provide another method for the confirmation of amplified product. However, a major disadvantage is that these nested reactions are prone to carryover contamination. Novel single-tube nested RT-PCR methods may help circumvent these issues. Ratcliff et al. (81) pooled the reagents required for the nested amplification in a "hanging drop" that could be introduced by centrifugation after the first RT-PCR amplification while Burkhardt et al. (14) compartmentalized the nested RT-PCR cocktail in a "tube-within-a-tube" device by using inexpensive materials such as a pipette tip and a microcentrifuge tube. Primers for the detection of hepatitis A virus by RT-PCR are summarized in Table 6.2.

ALTERNATIVE NUCLEIC ACID AMPLIFICATION METHODS

Nucleic acid sequence-based amplification (NASBA) is an amplification method that specifically detects RNA to the exclusion of DNA. The transcription-driven NASBA reaction is carried out at a single temperature (41°C) and theoretically amplifies the RNA target more than 10^{12}-fold within 90 min (34, 53). The final product of the amplification is single-stranded RNA, which can be readily detected by hybridization. The system utilizes three enzymes, (1) a reverse transcriptase (AMV-RT), (2) an RNase H, and (3) a T7 RNA polymerase, all of which act in a stepwise (sequential) manner with two oligonucleotide primers specific to the target (34, 53). One of the primers (P1) contains the T7 RNA

Table 6.1. Primers used in the detection of noroviruses in foods by RT-PCR

Primer	*Viruses*	*Sequence (5′→ 3′) (Polarity)*	*Location (bp)*	*Size (bp)*	*Food*	*Reference*
Calman-1	NV (GII)	GCACACTGTGTTACACTTCC	4193–4213	822	Clinical Stool	84
Calman-2		ACATTGGCTCTTGTCTGG	4997–5015			
MJV12	NV	TAY CAY TAT GAT GCH GAY TA	4553–4572	326	Shellfish	78
RegA		CTC RTC ATC ICC ATA RAA IGA	4859–4879			
Nested p290						
Mp290	NV	GAT TAC TCC AAG TGG GAC TCC AC	4568–4590	218	Shellfish	78
RevSR46		GAT TAT ACT SSM TGG GAY TCM AC				
RevSR48-52		CCA GTG GGC GAT GGA ATT CCA	4786–4766			
		CCA RTG RTT TAT RCT GTT CAC				
G1SKF	NV GI		5342–5362	330	Fecal	62, 112
GISKR		CTG CCC GAA TTT GTA AAT GA				
		CCA ACC CAR CCA TTR TAC A				
NI	NV GII	GAATTCCATCGCCCACTGGCT (+)	4756–4776	113	Shellfish	33, 37, 63
E3		ATCTCATCATCACCATA (−)	4869-4853			
P290	NVs and	GATTACTCCAAGTGGGACTCCAC (+)	4568–4590	319	Fruits and	32
P289	Sapoviruses	TGACAATGTAATCATCACCATA (−)	4865–4886		Vegetables	
NVLI410U24	NV GI	(T/C)TT(T/C)TC(A/T/C)TT (T/C)TA(T/C)GG (G/T)GATGATGA	4489–4512	450	Shellfish	65
NVLI839L20		GAA(G/C)CGCATCCA(G/A)CGGAACA				
NVLII184U23	Norovirus GII	CA(G/A)(T/C)GGAACTCCA(T/C) (T/C)(G/A)CCCACTG (+)	5044–5063	574	Shellfish	65
NVLII738L20		TGCGATCGCCCTCCCA(T/C)GTG (−)				
Mon381	Norovirus GII	CCAGAATGTACAATGGTTATGC (+)	5362–5383	322	Deli Sandwich	22

Continued

Table 6.1. Primers used in the detection of noroviruses in foods by RT-PCR—*cont'd*

Primer	*Viruses*	*Sequence (5′→ 3′) (Polarity)*	*Location (bp)*	*Size (bp)*	*Food*	*Reference*
Mon383	Nested with 381	CAAGAGACTGTGAAGACATCATC (−)	5661–5683	223	Shellfish	95
Mon382		TGATAGAAATTGTTCCTAACATCAGG (−)	5559–5584			
NVp110	Noroviruses	AC(A/T/G)AT(C/T)TCATCATCACCAA (−)	4865–4884	398	Clams	59
NVp36		ATAAAAGTTGGCATGAACA (+)	4487–4501			87
JV12	Noroviruses	ATA CCA CTA TGA TGC AGA TTA (+)	4552–4572	326	Lettuce and	24
JV13		TCA TCA TCA CCA TAG AAA GAG (−)	4858–4878		Shellfish	

[1] GI: Genogroup I Noroviruses
[2] GII: Genogroup II Noroviruses

Table 6.2. Primers used in the detection of hepatitis A virus in foods by RT-PCR

Primer	*Sequence (5′ → 3′) (Polarity)*	*Location (bp)*	*Size (bp)*	*Food*	*Reference*
H1	GGAAATGTCTCAGGTACTTTCTTTG (−)	2390–2414	247	Fruits/Vegetables	32
H2	GTTTTGCTCCTCTTTATCATGCTATG (+)	2167–2192		Mussels	101, 102
(HAVp3				Lettuce, Strawberry	12
HAVp4)				Shellfish	3, 4, 21, 70
(D, E)				Oysters	65, 69
				Delicatessen	91
HAV-R	CTCCAGAATCATCTCAAC (−)	2208–2226	192	Oyster	15, 16, 31, 51, 75
HAV-L	CAGCACATCAGAAAGGTGAG (+)	2035–2054		Lettuce, Hamburger	66, 87
				Mussel Tissue	82
2870	GACAGATTCTACATTTGGATTGGT (+)	2986–3004	534	Clams	59
3381	CCATTTCAAGAGTCCACACACT (−)	3381–3360			
BG7	CCGAAACTGGTTTCAGCTGAGG (−)	7125–7104	276	Produce and Shellfish,	35
BG8	CCTCTGGGTCTCCTTGTACAGC (+)	6850–6871		(Nested PCR)	
BG7a	CTGGTTTCAGCTGAGGYA (−)	7120–7102	264		
BG8a	GGTCTCCTTGTACAGCTT (+)	6856–6873			
2949	TATTTGTCTGTCACAGAACAATCAG (+)	2949–2973	267	Shellfish,	58
3192	AGGAGGTGGAAGCACTTCATTTGA (−)	3168–3145		(Nested PCR)	
dkA24	CTTCCTGAGCATACTTGAGTC (−)	3163–3182	200	Oysters	60
dkA25	CCAGAGCTCCATTGAACTC (+)	2986–3004			
Primer 1	CAGACTGTTGGGAGTGG (+)	762–778	385	Shellfish, (Nested PCR)	20
Primer 2	TTTATCTGAACTTGAAT (−)	1131–1147		Mussels, (Nested PCR)	19

Continued

Table 6.2. Primers used in the detection of hepatitis A virus in foods by RT-PCR—*cont'd*

Primer	*Sequence (5′ → 3′) (Polarity)*	*Location (bp)*	*Size (bp)*	*Food*	*Reference*
Primer 3	CAAGCACTTCTGTTTCCCCGG (+)	780–797	329		
Primer 4	ATTGTCACCATAAGCAGCCA(−)	1092–1109			
P1	CAGGGGCATTTAGGTTT (−)	669–685	415	Produce (Nested PCR)	18
P2	CATATGTATGGTATCTCAACAA (+)	1063–1084			
P3	TGATAGGACTGCAGTGACT (−)	807–825	211		
P4	CCAATTTTGCAACTTCATG (+)	1000–1018			
HAV240	GGAGAGCCCTGGAAGAAAGA (−)	194–213	170	Bivalve Molluscs	85
HAV68	TCACCGCCGTTTGCCTAG (+)	43–60			
HAV1	TTGGAACGTCACCTTGCAGTG (+)	332–353	368	Shellfish (Nested PCR)	76
HAV2	CTGAGTACCTCAGAGGCAAAC (−)	680–700			
neHAV1	ATCTCTTTGATCTTCCACAAG (+)	371–391	290		
neHAV2	GAACAGTCCAGCTGTCAATGG (−)	641–661			

polymerase promoter sequence at 5′ terminal and the other primer (P2) can be designed with a generic sequence that facilitates probe capture for amplicon detection by liquid hybridization (described in the Confirmation section below).

NASBA assays have been developed for the detection of foodborne enteric viruses such as hepatitis A virus, rotavirus and noroviruses. For instance, Greene et al. (41) applied the NucliSens Basic Kit NASBA protocol for the detection of norovirus RNA in stool using primers targeting the RNA polymerase region of the viral genome. Jean et al. (54, 55, 56) developed a NASBA-based method to detect hepatitis A virus on artificially contaminated lettuce and blueberry samples and also for the detection of human rotavirus. More recently, Jean et al. (53) developed a multiplex NASBA method for the simultaneous detection of hepatitis A and noroviruses (GI and GII) from lettuce and sliced turkey (deli meats).

The NASBA method is isothermal, as rapid (if not faster than) as RT-PCR, and demonstrates detection limits equal to if not better than RT-PCR (41, 53, 56). However, NASBA technology has many of the same limitations as RT-PCR (e.g., contamination control, sample volume considerations, matrix-associated reaction inhibitors). Nonetheless, it remains an important alternative method for the detection of foodborne viruses.

CONFIRMATION

Since nonspecific products of amplification are a major issue when food and environmental samples are tested, it is critical to confirm that the nucleic acid amplification products obtained are specific to the target. Most often, the confirmation step also improves the sensitivity of the assay. The most common confirmatory tool is Southern hybridization using specific oligoprobes internal to the amplicon. These probes are usually enzyme labeled for colorimetric, luminescent or fluorescent endpoints. When RNA products (for NASBA) are tested, Northern hybridization with labeled internal oligonucleotide probes may be used (45, 46). An oligonucleotide array dot-blot format for the simultaneous confirmation of norovirus amplicons and strain genotyping has recently been reported, offering the promise of providing both detection and strain typing in a single test (107).

DNA enzyme immunoassay (DEIA) methods provide an alternative to Southern hybridization. In these assays, a capture probe is immobilized to a microtiter plate well and a labeled amplicon can then be detected directly, or alternatively, an unlabeled amplicon can be hybridized to a second labeled detector probe followed by detection after the addition of an enzyme-conjugate and appropriate substrate (sandwich assay). For colorimeteric, luminescent, or fluorescent endpoints, absorbance is read using a conventional microtiter plate spectrophotometer or fluorescent plate reader. The intensity of the signal obtained may be approximately proportional to the concentration of amplicon. The sensitivity of the microtiter plate assay is generally equal to or better than Southern

hybridization and this approach has advantages including ease of interpretation, rapid (4 h) amplicon confirmation, and the potential for automation (54, 55, 90).

A liquid electrochemiluminescence (ECL) hybridization technology has been used commercially for the detection of NASBA amplicons. This technology utilizes two specific oligoprobes; a capture probe complementary to the sequence on primer P2, immobilized to streptavidin-labeled magnetic beads and a detector probe complexed to a ruthenium chelate. The hybridized magnetic particles are trapped on an electrode, and application of a voltage trigger to the electrode induces the ECL reaction such that the amount of emitted light is directly proportional to the amount of the amplicon. The signals are reported as ECL units by the NucliSens Reader and associated software. The NASBA-ECL system typically generates confirmed detection results in a day and has been used by Fox et al. (34) for the detection and confirmation of enterovirus from clinical samples and by Greene et al. (41) and Jean et al. (53) for the detection Norwalk virus in stool and food samples.

Other confirmation methods include specific "nested" PCR reactions (36, 43, 63, 99), which use a second pair of primers internal to the first amplicon sequence; and restriction endonuclease digestion of RT-PCR products (36, 43). Direct sequencing of the amplicon for the confirmation of RT-PCR products is another method of choice, and is frequently applied in the clinical realm, and more recently when amplicons are obtained from foods implicated in outbreaks (69, 91).

REAL-TIME DETECTION

Real-time detection refers to the simultaneous detection and confirmation of amplicon identity as the amplification reaction is progressing, thereby linking nucleic acid amplification with hybridization. There are currently five main chemistries used for real-time amplification and detection. One of the earliest and simplest approaches to real-time PCR, called DNA binding fluorophores, uses ethidium bromide or SYBR green I compounds that fluoresce when associated with double stranded DNA and exposed to a suitable wavelength of light. These methods tend to lack specificity but this has been addressed recently by coupling the assay with melting curve analysis. The 5′ endonuclease assay (e.g., TaqMan oligoprobes, Applied Biosystems, Foster City, CA), adjacent linear probes (e.g., HybProbes, Roche Molecular Biochemicals, Germany) and hairpin oligoprobes (e.g. molecular beacons, Molecular Probes, Eugene, Oregon) have received considerable attention of late. Self-fluorescing amplicons (e.g., Sunrise primers, Amplifluor hairpin primers, Intergen Co., Purchase, NY) incorporated into the PCR product as the priming continues have a 3′ end complimentary to the target strand and Scorpion primers have 5′ end complementary to the target strand) (reviewed in 72). Recently, investigators have developed real-time PCR systems for the detection of a wide array of bacterial pathogens in foods. Prototype real-time RT-PCR amplification technologies have been developed for the detection of hepatitis A virus (17) and

noroviruses (79) using the TaqMan format, and the norovirus detection uses the SYBR Green melting curve format (73). Beuret et al. (10) have used multiplex real-time PCR for the simultaneous detection of a panel of enteric viruses. Research efforts are currently underway to apply these methods to the detection of viruses in food matrices. Indeed, Myrmel et al. (78) recently reported the detection of viral contamination in shellfish using a commercial SYBRGreen PCR kit, while Narayanan et al (79) used their TaqMan assay to detect noroviruses in shellfish.

CONCLUSIONS

It should be clear from the preceding discussion that the current methodology for the detection of enteric viral contamination in foods is less than ideal and that research is necessary to improve these methods. Indeed, these protocols are applied infrequently and usually only in response to known or suspected foodborne disease outbreaks. The most important reasons for their limited use include: (1) the inability of molecular amplification methods to discriminate between infectious and inactivated virus, (2) the lack of widely accepted, collaboratively tested methods, (3) the requirement that most methods be product specific, meaning that universal approaches do not exist, and (4) the cost and need for highly trained personnel (83). When taken together, detection limits ranging from approximately 1–100 infectious units/g food have been obtained using various RT-PCR methods. The use of internal amplification standards to simultaneously evaluate RT-PCR inhibition and/or to provide a semiquantitative assay is also frequently done (5, 6, 67, 69, 89, 93, 103).

The failure to discriminate between infectious and inactivated virus is of critical importance because the inactivated forms of these pathogens pose no real public health threat. There is also a need to develop more universal sample extraction methods. For the most part, virus concentration from foods is likely to remain product dependent but research is needed to develop and refine the prototype methods into collaboratively tested protocols. Researchers continue to seek efficacious methods to concentrate the pathogens from the food matrix with the simultaneous removal of matrix-associated inhibitors. Even so, the methods will probably never be perfect and will always require a high degree of sample manipulation by the laboratory personnel (83). There is hope, however, that over time these rapid methods to detect human enteric viruses in foods may become more widely available to the food safety community.

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