CHAPTER 5

Molecular Methods of Virus Detection in Foods

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1.0. INTRODUCTION

Food-borne transmission of virus infections has been recognized for more than 5 decades (Svensson, 2000). The principal clinical syndromes associated with food-borne viruses are hepatitis and gastroenteritis, but not all enteric viruses (Table 5.1) have been linked to food-borne illness either epidemiologically or by direct pathogen detection. The primary means of identifying viruses as causes of food-borne outbreaks has been through the recognition of a common viral pathogen in consumers and the use of epidemiologic methods to identify a particular food as the vector. More direct methods, such as the detection of viruses in food, have largely been unsuccessful because only small quantities of viruses are generally present in food, and these viruses are either difficult to grow in cell cultures or are noncultivable.

In the past two decades, molecular assays have been developed for the detection of a number of pathogens, including food-borne viruses. In the past decade, the sensitivity of these assays has improved to the point that their application to virus detection in foods can be considered. This chapter will provide an overview of molecular assays that are available for pathogen detection, examples of application of these methods for the detection of viruses associated with food-borne illness, and the limitations of these assays.

2.0. NONAMPLIFICATION METHODS (PROBE HYBRIDIZATION)

Probe hybridization assays were the first molecular assays applied to the detection of enteric viruses. In these assays, single-stranded RNA or DNA probes that are complementary to a viral genomic sequence are linked to a reporter (radioisotope, enzyme, chemiluminescent agent) and hybridized with the target. The probes can range in size from 15 to 20 to several hundred nucleotides. 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 hybridization, the unbound probe is washed away and the bound probe is

Table 5.1 Enteric Viruses and Their Association with Food-borne Illness

Virus	*Family*	*Disease*	*Food-borne Transmission Demonstrated*
Enteric adenovirus	Adenoviridae	Gastroenteritis	No
Astrovirus	Astroviridae	Gastroenteritis	Yes
Human caliciviruses	Caliciviridae		
Norovirus		Gastroenteritis	Yes
Sapovirus		Gastroenteritis	Yes
Hepatitis A virus	Picornaviridae	Hepatitis	Yes
Hepatitis E virus	Unclassified	Hepatitis	Yes
Rotavirus	Reoviridae	Gastroenteritis	Yes

detected by fluorescence, radioactivity, or color development. In liquid-phase hybridization, both the target and probe are in solution at the time of hybridization. Probe signal can then be detected by fluorescence or color change. *In situ* hybridization is used to detect target nucleic acids within an infected cell. The principal application of this method for the detection of viruses in foods would be to couple the hybridization assay to a cell culture system (Jiang et al., 1989). Because the method is more cumbersome than antigen detection methods, it is rarely used for this purpose.

Probe hybridization assays have been described for the detection of a number of enteric viral pathogens (Dimitrov et al., 1985; Jansen et al., 1985; Takiff et al., 1985; Willcocks et al., 1991). However, their sensitivity is no better than that of antigen detection methods (approximately 10,000 genomic copies) (Jiang et al., 1992). Thus, this is not a practical method for the detection of enteric viruses in foods but can be incorporated into some of the other molecular methods described below to confirm their specificity.

3.0. AMPLIFICATION METHODS

The era of molecular diagnostics began with the development of methods to detect low numbers of pathogens in clinical and environmental samples. Since the initial description of the polymerase chain reaction (PCR) assay (Saiki et al., 1985), a number of different strategies have been developed for use in molecular assays (Table 5.2). These methods are based on amplification of the target nucleic acids, amplification of the signal generated after probe hybridization, and amplification of a probe sequence (Nolte and Caliendo, 2003). One or more examples of each are described in the following sections.

3.1. Target Amplification

Several target amplification systems have been described, with PCR-based assays being the best known and most commonly used. In each of these

systems, enzymatic reactions are used to amplify a portion of the target nucleic acid 1 million–fold or more to the point that the amplified products can be easily detected and analyzed. One of the major pitfalls of these strategies is that the products generated can serve as a contaminating template for subsequent assays and lead to false-positive results. Strategies to prevent carryover contamination are addressed later in this chapter.

3.1.1. PCR

In its simplest form, the PCR uses a DNA polymerase to amplify a DNA template. The core components of this chemical reaction are the DNA polymerase, equimolar concentrations of deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP), molar excess of two oligonucleotide primers, and an appropriate buffer. The oligonucleotide primers are complementary to sequences on opposite strands of the target template, and these primers flank the region to be amplified. There are three basic steps that are repeated through a variable number of cycles: (1) heat denaturation, (2) primer annealing, and (3) primer extension. The initial step denatures double-stranded DNA into single-stranded DNA using heat (92°C and 95°C). The reaction mix is then rapidly cooled to 40–60°C when oligonucleotide primers preferentially bind to the single-stranded DNA template because they are present in a much higher concentration than the template. In the third step, the DNA polymerase adds nucleotides to the 3′ end of the primer that are complementary to the sequence of the template (Fig. 5.1). This step occurs

Table 5.2 Common Nucleic Acid Amplification Methods Used for Pathogen Detection and Application to Enteric Viruses

Method	*Amplification Strategy*	*Potential for Carryover Contamination*	*Assay Described for Enteric Viruses*	*Assay Applied to Detect Enteric Viruses in Food*
Polymerase chain reaction (PCR)	Target amplification	Yes	Yes	Yes
Nucleic acid sequence-based amplification (NASBA)	Target amplification	Yes	Yes	Yes
Strand displacement amplification (SDA)	Target amplification	Yes	No	No
Branched DNA (bDNA) amplifcation	Signal amplification	No	No	No
Ligase chain reaction (LCR)	Probe amplification	Yes	No	No

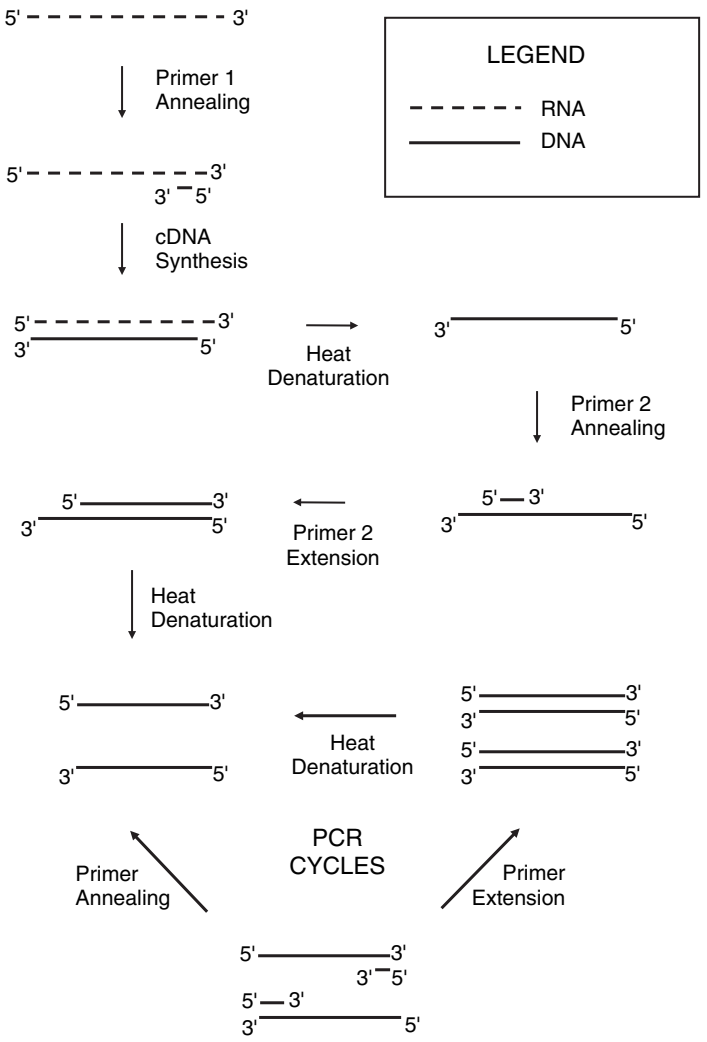

Figure 5.1 Schematic of RT-PCR reaction. Primer 1 anneals to the RNA, and cDNA is synthesized by reverse transcriptase. After denaturation of the RNA:cDNA hybrid, primer 2 anneals to the cDNA and is extended to form a second strand. This double-stranded DNA then enters cycles of heat denaturation, primer annealing, and primer extension. The number of copies of amplicons can double with each cycle if the reaction occurs at maximal efficiency.

at a temperature (70–75°C) that is optimal for the enzymatic activity of the DNA polymerase.

The three steps of heat denaturation, primer annealing, and primer extension are repeated in each cycle. If the reaction proceeds with absolute efficiency, the amount of amplified DNA doubles after each cycle. Thus, the

target DNA sequence can be amplified approximately 1 million (2^{20})-fold after 20 cycles. In practice, the efficiency of PCR amplification is less than ideal especially with increases in the number of cycles.

The initial description of the PCR assay used a thermolabile DNA polymerase (Klenow fragment of *Escherichia coli* DNA polymerase), and hence the enzyme had to be replaced after each heat denaturation step (Saiki et al., 1985). The identification of a thermostable DNA polymerase prevented heat inactivation of the enzyme after each cycle and allowed the automation of the amplification reaction using programmable thermal cyclers (Saiki et al., 1988). Since the initial description of Taq polymerase derived from *Thermus aquaticus*, additional thermostable DNA polymerases have been described. Some of the newer thermostable DNA polymerases have higher fidelity (lower error rates) than the Taq polymerase (Cline et al., 1996).

3.1.2. RT-PCR

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, a complementary DNA (cDNA) is synthesized, which is then amplified by PCR using the same steps as described above for a DNA 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), or it can be random hexamers or oligo-dT (if the genomic region to be amplified is near a polyadenylated site). A two-step RT-PCR assay is the one in which 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.

Most RT-PCR assays use a heat-labile reverse transcriptase, such as avian myelobastosis virus (AMV) reverse transcriptase or Moloney murine leukemia virus (MMLV) reverse transcriptase, and the cDNA synthesis step is carried out at a lower temperature (<50°C). The development of a thermostable DNA polymerase from *Thermus thermophilus* that has both reverse transcriptase and DNA polymerase activities has allowed the use of a single enzyme for RT-PCR assay (Myers and Gelfand, 1991). This enzyme has been successfully incorporated into assays used to detect noroviruses in shellfish, and the limits of virus detection are similar to assays using the Taq polymerase (Schwab et al., 2001). However, subsequent studies have demonstrated that this enzyme fails to adequately amplify viral genome with certain primer pairs that can be used successfully with Taq polymerase.

3.1.3. Nested PCR

Nested PCR is the serial amplification of a target sequence using two different primer pairs (Haqqi et al., 1988). The initial amplification is performed using an outer primer pair and 15–30 cycles of amplification. A second round of amplification is then performed using primers that anneal to a region

located (or nested) between 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.

Nested PCR also has the potential to increase the specificity of the assay because both primer pairs must amplify the target sequence. However, the major problem with this approach is the potential for carryover contamination. PCR products from the first round of amplification may contaminate the laboratory or cross-contaminate other tubes during their transfer to a second tube for the second round of amplification. The cross-contamination causes false-positive results and thus can decrease the specificity of the assay. Although carryover contamination is a potential problem for any PCR assay, the methodologic controls (see Section 6.0) used to prevent this problem are more likely to fail for nested assays. Nevertheless, strategies that do not require reopening the tube after the first round of amplification have been developed (Ratcliff et al., 2002). In the "hanging drop" method, reagents (inner primer pair, additional DNA polymerase) for the nested PCR step are placed in the cap of the tube and are added to the overall reaction mix by centrifugation after the first round of PCR is complete.

3.1.4. Multiplex PCR

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 the evaluation of a sample for more than one virus at a time and can also identify the presence of multiple viruses in a single reaction. However, the primers for different targets should have similar annealing temperatures and lack complementarity so that each target can be efficiently amplified. Even when such steps are taken, the multiplex PCR assays usually have decreased sensitivity as compared with the standard PCR assays due to competition for reagents. A high concentration of one target (virus) can prevent the detection of other targets present in lower concentrations that would have been detected if the high concentration target was absent. This can thus lead to false-negative results for the lower concentration target.

3.1.5. Postamplification Analysis

After PCR amplification of a target nucleic acid, additional analyses must be performed to interpret the results of the assay as outlined in Table 5.3. The simplest method is to perform gel electrophoresis using all or a portion of the PCR reaction mix. Molecular weight markers are run concurrently and the presence of a band of the expected amplicon product size (based on the genomic location targeted by the primers) is intepreted as a positive result. Although this approach is simple, the occurrence of nonspecific amplification can lead to bands that are of the expected size but are not virus-specific (Atmar et al., 1996). This approach is applied most commonly with nested

Table 5.3 Postamplification Analysis Strategies for Target Amplification

1. Gel electrophoresis
2. Restriction
3. Hybridization
   (a) Solid phase
       Slot/dot blot
       Southern blot
       Microarrays
   (b) Liquid phase
4. Sequencing

PCR assays, but caution must be used because this amplification strategy can result in nonspecific amplification. One of the following approaches may provide additional reassurance as to the specificity of the assay.

Restriction analysis combines gel electrophoresis with digestion of virus-specific amplicons using a restriction endonuclease. With this strategy, amplicons of the expected size must be generated, and the amplicons must have a specific restriction site. Digestion of the virus-specific amplicons leads to the generation of shorter fragments whose size can be predicted based on the location of the restriction site. Generation of bands of the expected sizes after restriction is interpreted as a positive result.

Hybridization (see Sec. 2.0) is the most common approach used to identify and confirm a positive PCR result. Many different hybridization formats are used, including dot/slot blots, Southern blots, and liquid hybridization. Southern blot hybridization has the advantage of providing information about the product size in addition to reactivity with a virus-specific probe. The disadvantage of Southern blot hybridization is the additional time and effort required to perform the assay. This method usually adds a day to the overall assay. In contrast, liquid hybridization assays can yield results within 1 hr after the completion of the PCR step.

Hybridization assay formats have been developed that allow multiple probes to be used. The reverse line blot arrays oligonucleotide probes on a solid matrix (e.g., nylon membrane), and these probes are hybridized to denatured amplicons. One of the primers used during the amplification process is biotinylated and hybridization of the strand containing the biotinylated primer to a virus-specific probe is detected using a streptavidin reporter, such as a peroxidase enzyme that can react with an appropriate substrate. This method has been used to not only identify virus-specific amplicons but also to further characterize norovirus strains using genotype-specific probes (Vinjé and Koopmans, 2000). This technology can be taken further with the use of DNA microarrays. Hundreds or thousands of probes are fixed to a surface (such as a silica wafer or glass slide) and hybridization of labeled products to specific probes is detected (Nolte and Caliendo, 2003). Microarrays have been used in combination with RT-PCR for the detection and

genotyping of group A rotaviruses (Chizhikov et al., 2003; Lovmar et al., 2003). With a large enough number of probes, it should even be possible to deduce the sequence of virus-specific amplicons based on the hybridization patterns. However, improvements in the current technology are needed to decrease the complexity and costs of microarrays so that their potential can be fully realized (Nolte and Caliendo, 2003).

The development and increased availability of automated sequencers have led to the use of direct sequencing of amplicons as a measure of specificity. The sequence data can provide information not only confirming the specificity of the amplification but also for genotyping or classifying virus strains (Robertson et al., 1991). The information can be combined with epidemiologic data for use in surveillance and outbreak investigations (Koopmans et al., 2003). A greater quantity of amplicons is needed to generate sequence data, making this method less sensitive than the hybridization techniques described above.

3.1.6. Real-Time PCR

Automated instruments have been developed to allow the specific detection of amplified nucleic acids in a closed system. Real-time PCR can improve the efficiency of the analytic process while decreasing the risk of carryover contamination by eliminating the need for post-PCR manipulation of amplicons in confirmatory tests of specificity. Disadvantages include the capital expense of the equipment (thermal cycler and amplicon detection equipment), limited ability to perform multiplex assays, and the inability to monitor the amplicon size (Mackay et al., 2002).

There are two principal approaches for the detection of amplified products: use of DNA-binding fluorophores (fluorescing dyes) and use of specific oligoprobes. The fluorophores intercalate with double-stranded DNA and fluoresce after exposure to a specific wavelength of light. SYBR Green is the most commonly used fluorophore, but ethidium bromide and YO-PRO-1 are also used. A melting curve analysis is used to distinguish virus-specific amplicons from nonspecific primer-dimers with the former having higher dissociation temperatures. The utility of fluorophores for amplicon detection is limited by the inability of this approach to identify amplicons that result from nonspecific amplification in the initial PCR steps, especially when the target is present in low concentrations (as might be expected in contaminated foods) (Mackay et al., 2002).

Fluorescently labeled oligoprobes are the principal means for specific amplicon detection in real-time PCR assays (Fig. 5.2). The two most common methods use oligoprobes that are dual-labeled with a reporter fluorophore and a quencher fluorophore. As the name implies, the quencher fluorophore will quench the signal from the reporter fluorophore when the two are in close proximity and are exposed to a certain wavelength of light (also called fluorescence resonance energy transfer, or FRET). 5′ nuclease oligoprobes have the reporter fluorophore on the 5′ end of the oligoprobe and the quencher fluorophore on the 3′ end, and their melting temperature is gen-

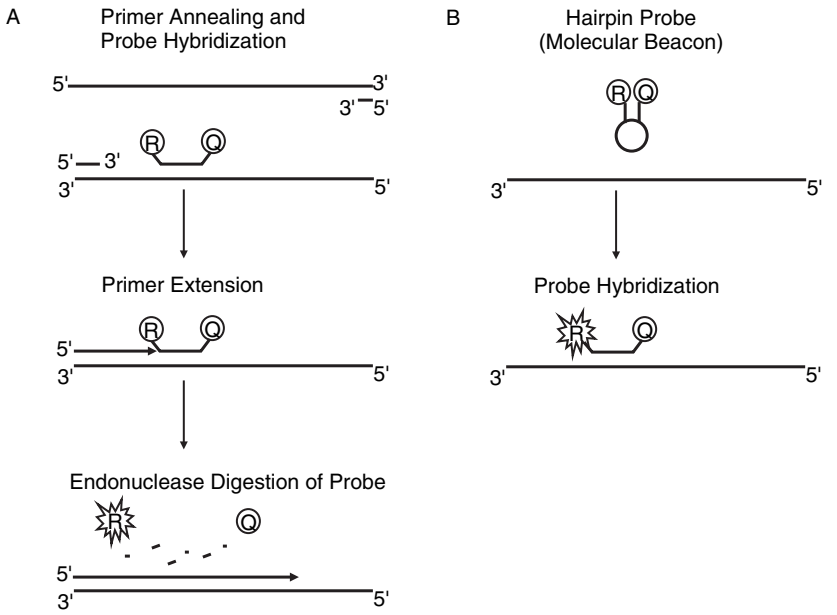

Figure 5.2 Schematic of fluorescent probe detection of PCR products in real-time PCR assays. (A) 5′ nuclease oligoprobes. A probe with a fluorescent reporter (R) and quencher (Q) hybridizes with the target amplicons strand. The quencher suppresses the signal generated by the reporter. The strand-specific primer is extended during the PCR reaction by the polymerase, and the exonuclease activity of the polymerase separates the reporter from the quencher, allowing increased fluorescent signal to be generated. (B) Hairpin oligoprobes or molecular beacons. The fluorescent reporter (R) and quencher (Q) are juxtaposed by hybridization of the 5′ and 3′ ends of the probe, allowing the quencher to suppress the signal of the reporter. Hybridization of the probe to the target physically separates the reporter and quencher, allowing an increase in the reporter's fluorescent signal.

erally ~10°C higher than those of the primers used for amplification. If virus-specific amplicons are generated, the oligoprobe will hybridize to its target sequence and the 5′-3′ exonuclease activity of the Taq polymerase will release the reporter fluorophore by hydrolysis. Fluorescence from the freed reporter fluorophore can then be detected and as the reaction proceeds, the amount of fluorescence increases proportional to the amount of target amplicons generated. Hairpin oligoprobes, or molecular beacons, are the other commonly used dual-labeled probe. The probes are designed to form a hairpin structure so that the reporter and quencher fluorophores are juxtaposed in the absence of a specific DNA target. When a target sequence with a complementary sequence is present, the probe can hybridize and assume a linear conformation. Fluorescent signal can then be detected after the spatial separation of the quencher from the reporter fluorophore (Mackay et al., 2002).

3.1.7. Application to Food-borne Viruses

PCR assays have been developed for food-borne viruses listed in Table 5.1. The utility of these assays has varied based on the availability of other diagnostic tests. PCR assays for each of these viruses are described in the following paragraphs.

The adenovirus hexon gene is the most common target of PCR assays for these viruses, although other viral genes have also served as targets (Allard et al., 1992; Xu et al., 2000). Enteric adenoviruses (group F) can be further differentiated from nonenteric adenoviruses through the use of group-specific primers, postamplification restriction analysis, and hybridization analyses (Rousell et al., 1993; Allard et al., 1994; Soares et al., 2004). To date, no real-time PCR assays for enteric adenoviruses have been developed although such assays are described for nonenteric adenoviruses (Gu et al., 2002). The sensitivity of these assays is similar to that of antigen detection assays so that the general use of PCR for the detection of adenoviruses in clinical and environmental samples is limited.

RT-PCR assays are the most sensitive means of detecting astroviruses. Nonstructural genes (putative protease region [ORF1a] and RNA-dependent, RNA polymerase region [ORF1b]) and the 3′ noncoding region are the most common regions of the viral genome targeted for amplification, although the capsid gene (ORF2) has also been targeted, especially when further characterization of strains within a serotype is desired (Jonassen et al., 1995; Noel et al., 1995; Belliot et al., 1997). Passage in cell cultures for up to 48 hr can expand the quantity of virus in a sample prior to amplification (integrated cell culture/RT-PCR), and this approach may improve sensitivity of detection as compared with that of RT-PCR alone (Mustafa et al., 1998). Although real-time RT-PCR assays for astrovirus have been described, additional studies are needed to evaluate the performance characteristics of these assays (Grimm et al., 2004; Le Cann et al., 2004).

RT-PCR assays are now the primary means of diagnosis of human calicivirus infections, supplanting the prior use of electron microscopy (Atmar and Estes, 2001). The RNA-dependent RNA polymerase and the capsid genes are the primary targets for amplification, although primers that amplify other regions of the genome have also been described (Matsui et al., 1991). Despite the evaluation of many different primer sets, no single detection assay is able to detect all strains because of the genetic diversity of these viruses (Atmar and Estes, 2001; Vinjé et al., 2003). Most laboratories will select one or a few primer sets (e.g., different pairs for genogroup I and II noroviruses and for sapoviruses) for use in initial screening of samples. If no virus is detected with the selected primers but the index of suspicion for the presence of caliciviruses remains high, the use of additional primer pairs can lead to successful virus detection (Le Guyader et al., 2004). Nested RT-PCR is also used in some laboratories (Schreier et al., 2000). Real-time RT-PCR assays that use probe detection or SYBR Green for amplification detection are also available for noroviruses, although the general utility of these assays remains to be determined (Kageyama et al., 2003; Pang et al., 2004a). The

genetic diversity of noroviruses is likely to adversely affect the utility of real-time RT-PCR assays for virus quantitation because base mismatches between the primers and target sequence will decrease the efficiency of amplification. The number of strains detected may also be limited as seen with standard RT-PCR assays.

Hepatitis A virus was one of the first enteric viruses for which an RT-PCR assay was developed (Jansen et al., 1990). Many human strains can be amplified using a single primer set, although nested PCR assays have been used to increase assay sensitivity (Robertson et al., 1991; Hutin et al., 1999). Genes of the structural proteins (VP1-2A junction; VP3-VP1 junction) are most commonly targeted for amplification, although the 5′ noncoding region of the viral genome is also used (Pina et al., 2001). Real-time RT-PCR assays are available as is an integrated cell culture system, but the utility of these assays is still under investigation (Abd El Galil et al., 2002; Jiang et al., 2004). Because hepatitis A virus (HAV) is difficult to cultivate and grows very slowly in cell cultures, RT-PCR assays now are considered to be the best means of direct HAV detection.

Initial studies on hepatitis E virus (HEV) suggested little genetic heterogeneity among these strains, but only a limited number of strains had been analyzed. Use of degenerate primers and progressive decrease in the annealing temperature during PCR amplification (touchdown PCR) have allowed the amplification of additional strains of HEV and have demonstrated more genetic diversity among these viruses than previously realized (Schlauder et al., 2000; Schlauder and Mushahwar, 2001). More recently, an RT-PCR assay was designed to amplify all known strains, but thus far it has been validated with only a limited number of strains (Grimm and Fout, 2002). A real-time RT-PCR assay has also been described using SYBR Green detection of amplified products, but it has only been evaluated using a single HEV strain (Orrù et al., 2004). Additional studies are needed to determine the utility of these assays for the detection of HEV.

Rotaviruses are double-stranded RNA viruses, and the performance of RT-PCR assays is complicated by difficulties in denaturing the genome for the cDNA synthesis step. Dimethyl sulfoxide (DMSO) is often used in the reverse transcription step to help denature the double-stranded RNA. This approach has been used in assays developed for the detection of human rotavirus groups A, B, and C (Gouvea et al., 1990, 1991). These assays have not proved to be much more sensitive than the ELISA tests, although the performance characteristics of PCR can be improved when used in a nested or semi-nested format and when strain-specific oligonucleotides are used for cDNA synthesis (Gouvea et al., 1990; Buesa et al., 1996; Iturriza-Gomara et al., 1999). RT-PCR assays also are used to genotype strains, and the results correlate very well with serotyping assay results (Gouvea et al. 1990; Gentsch et al., 1992). Most of the RT-PCR assays developed and described in the 1990s target the structural genes VP4, VP6, and VP7 and amplify large segments of the genes. More recently, a real-time assay that amplifies an 87-base-pair segment of the nonstructural protein-3 (NSP-3) gene was described,

which achieves a diagnostic sensitivity comparable to that seen with nested PCR assays without the risk of cross-contamination inherent in nested assays (Pang et al., 2004b). Additional studies with this assay are needed to determine how many different strains can be recognized, as only a limited number of G-types (G1, G2, and G4) have been evaluated and detected.

3.2. Transcription-Based Amplification

Transcription-based amplification systems represent another approach to detecting viruses by amplifying a portion of the genome and then detecting the amplified products. Two variations of transcription-based amplification systems have been described: nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA). NASBA is the principal method that has been developed thus far for the detection of enteric viruses, so this method will be described below while noting the methodologic differences between NASBA and TMA.

3.2.1. NASBA

NASBA is performed at a single temperature (isothermal) and uses two virus-specific primers, avian myeloblastosis virus (AMV) reverse transcriptase, RNase H, and T7 RNA polymerase in the reaction tube. The NASBA reaction leads to the generation of single-stranded RNA transcripts that are then detected by probe hybridization. In the initial step (Fig. 5.3), one of the virus-specific oligonucleotides that also contains a T7 promoter sequence at its 5′ end binds to the RNA target and primes the synthesis of a cDNA by the AMV reverse transcriptase. RNase H digests the RNA in the cDNA:RNA hybrid, and the second virus-specific oligonucleotide binds to the cDNA and primes the synthesis of a second strand of DNA using the DNA-dependent DNA polymerase activity of the reverse transcriptase. The T7 RNA polymerase recognizes the double-stranded DNA promoter region and generates RNA transcripts that can then feed back into a continuous cycle of cDNA synthesis, RNase H digestion, second-strand synthesis, and RNA transcript production. Up to a billion-fold amplification of the target RNA can be attained within two hr (Nolte and Caliendo, 2003).

3.2.2. TMA

The principal difference between NASBA and TMA is that TMA uses a reverse transcriptase with endogenous RNase H activity, whereas in NASBA the AMV reverse transcriptase lacks RNase H activity and this enzyme must be added separately.

3.2.3. Application of NASBA to Food-borne Viruses

The use of NASBA assays for the detection of enteric viruses is less well studied than the RT-PCR assays. NASBA assays have only been described for hepatitis A virus, noroviruses, astroviruses, and rotaviruses (Jean et al., 2001, 2002; Greene 2003; Tai et al., 2003; Moore et al., 2004). However, the sensitivity of these assays is as good or better than comparable RT-PCR assays (Jean et al., 2001; Greene et al., 2003; Tai et al., 2003). NASBA assays

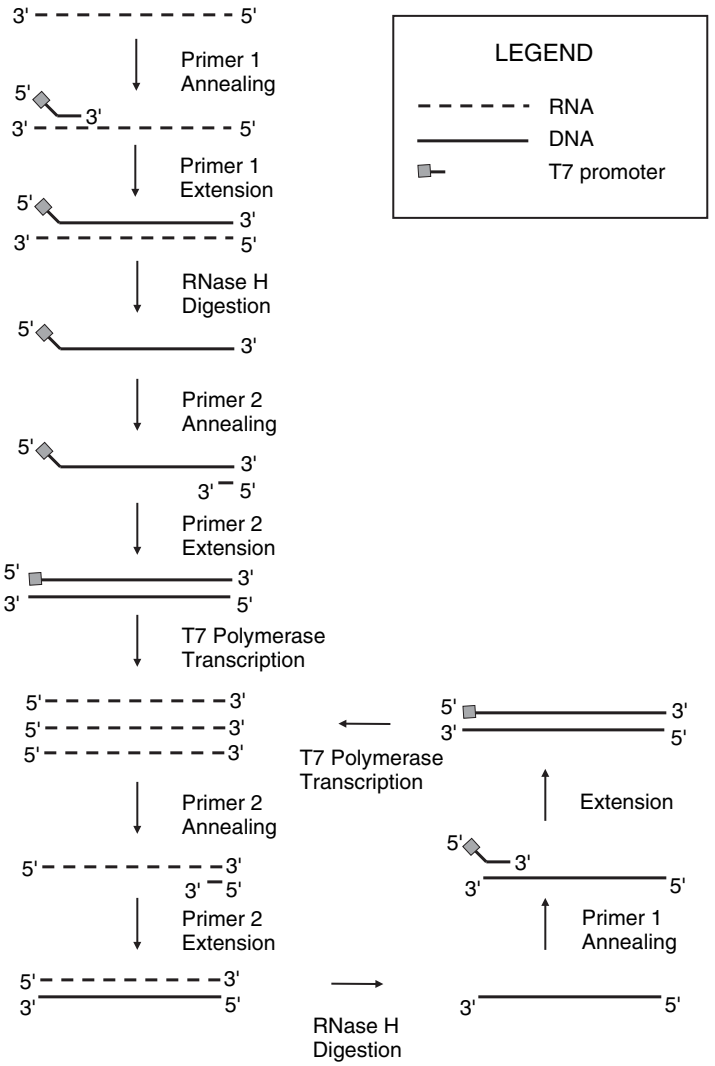

Figure 5.3 Schematic of NASBA reaction. Primer 1 anneals to the target RNA, and cDNA is made by reverse transcriptase. RNase H degrades the RNA strand, and primer 2 anneals to the cDNA and is extended by the polymerase activity of the reverse transcriptase. The T7 promoter is recognized by the T7 polymerase, which then generates numerous RNA transcripts. Primer 2 anneals to the RNA transcript, and cDNA is synthesized by the reverse transcriptase. RNase H degrades the RNA strand. Primer 1 anneals to the cDNA target, a second strand of DNA is synthesized, and T7 polymerase generates more RNA transcripts.

can also be multiplexed or coupled to RT-PCR assays to enhance virus detection (Jean et al., 2002, 2003, 2004). No TMA assays for enteric viruses have been described, and the overall utility of this approach remains to be determined. The simplicity, rapidity, and sensitivity of NASBA assays compared with RT-PCR assays suggest that further development of this approach should be pursued.

3.3. Other Signal Amplification Methods

Strand displacement amplification (SDA) and loop-mediated isothermal amplification (LAMP) are two additional target amplification methods that are available for viral diagnostics (Walker et al., 1992; Notomi et al., 2000). Both methods are based on amplification of DNA using a DNA-dependent DNA polymerase and at least four virus-specific primers. Thus, a cDNA must first be made from RNA targets. Although neither of these methods has been applied to enteric viruses or to detection of pathogens in foods, they are able to detect low numbers of nucleic acid targets as is possible with RT-PCR and NASBA (Parida et al., 2004).

3.4. Signal Amplification

Although target amplification systems are able to detect low copy numbers of a virus, issues related to carryover contamination leading to false-positive results have plagued these assays. A strategy to circumvent this problem has been the development of signal amplification systems in which neither the target nor the probe is amplified. Instead, the amount of signal generated is directly proportional to the amount of target nucleic acid. Several reporter molecules are present for each copy of target nucleic acid. This approach has the additional advantage of not being dependent on enzymatic reactions and thus is not subject to enzyme inhibitors that may be present in a sample (Nolte and Caliendo, 2003).

The principal method of signal amplification that is in use is the branched DNA (bDNA) assay, although to date no assays have been developed for any of the food-borne viruses. Oligonucleotide probes anchored to a solid substrate capture the target nucleic acid (RNA or DNA) (Urdea et al., 1991). Additional target-specific probes also bind the target. The second set of target-specific probes also binds to preamplifier molecules that will bind to bDNA amplifiers. Each bDNA amplifier then hybridizes to conjugate-labeled probes. With this approach, the signal is amplified several hundred-fold compared with a simple hybridization assay. The ease of assay performance, simple sample preparation method, and quantitative results obtained suggest that the development of this approach for the detection of food-borne viruses is warranted.

3.5. Probe Amplification

Probe amplification is a third strategy for the detection of a target nucleic acid. The only sequences contained in amplification products are those that were present in the original probe. The ligase chain reaction (LCR) is the

most well developed of the probe amplification methods. In LCR, two target-specific oligonucleotide probes hybridize to adjacent sequences forming a nick. A thermostable DNA ligase closes the nick, joining the 3′ end of one oligonucleotide to the 5′ end of the other nucleotide. Two additional oligonucleotides that recognize the complementary strand of the original target as well as the ligated product generated in the initial ligation reaction are also added to the reaction and can be ligated together in subsequent cycles. Thus, logarithmic amplification can occur with each cycle of denaturation, annealing, and ligation (Wu and Wallace, 1989). The oligonucleotides are labeled with haptens so that one hapten will be captured on a solid substrate and the other will react with an antibody conjugate. Signal generated by the conjugate will be present only if the two oligonucleotides are ligated (Nolte and Caliendo, 2003). Because ligation products can serve as template, this probe amplification method is subject to carryover contamination similar to that seen with target amplification methods. No probe amplification methods have been developed for any of the food-borne viruses.

4.0. SPECIMEN PREPARATION

The goal of specimen preparation is to concentrate and purify viral nucleic acids from the sample being analyzed so that even small amounts of virus can be detected. Because many of the molecular assays described above require the use of functional enzymes (e.g., Taq polymerase, reverse transcriptase), substances that inhibit enzymatic activity can lead to false-negative results. Thus, a major objective of the specimen preparation process is the removal of PCR inhibitory substances. The steps used in sample preparation generally consist of one or more of the following (Table 5.4): (1) elution of the virus from food; (2) extraction with an organic solvent; (3) concentration of the virus; and (4) extraction of viral nucleic acids. Each of these steps is discussed below.

4.1. Elution

One of the first steps in many sample processing protocols is to separate the virus from the food matrix using an elution procedure. In foods that have been contaminated superficially, virus can be eluted by simply rinsing the food with a saline solution or glycine buffer (Schwab et al., 2000; Jean et al., 2004; Le Guyader et al., 2004b). In most other circumstances, the food is homogenized prior to the elution procedure. Virus may then be eluted directly from the food matrix using either a direct elution procedure or an adsorption-elution method.

A variety of buffers and solutions have been used successfully in direct elution protocols, including solutions of glycine and sodium chloride, borate and beef extract, saline and beef extract, and beef extract alone (Lees et al., 1994; Traore et al., 1998; Leggitt and Jaykus, 2000; Sair et al., 2002; Le Guyader et al., 2004b). Only a few studies have reported direct comparisons of dif-

Table 5.4 Major Steps in Concentration and Extraction of Viral Nucleic Acids

1. Elution
Beef extract ± saline or borate
Borate
Glycine/NaCl buffer (high pH)
Saline or phosphate-buffered saline
2. Organic solvent extraction
Choroform: butanol
Freon
3. Concentration
Antibody capture
Ligand capture
Organic flocculation
Polyethylene glycol (PEG) precipitation
Ultracentrifugation
4. Nucleic acid extraction
Guanidinium isothiocyanate (GITC)
Phenol : chloroform

ferent eluting buffers but, in these reports, glycine-based buffers have generally performed better than the other solutions tested (Traore et al., 1998; Le Guyader et al., 2004b).

Adsorption-elution also is used for virus extraction from foods. With this method, the pH and solution conductivity are lowered to adsorb the virus to solids in the homogenate followed by elution of virus using a glycine/sodium chloride buffer (pH 7.5). Adsorption-elution has primarily been applied to shellfish but has also been used successfully to detect enteric viruses in blueberries (Jaykus et al., 1996; Shieh et al., 1999; Calder et al., 2003).

4.2. Organic Solvent Extraction

Extraction with an organic solvent can be used to remove organic compounds that are insoluble or poorly soluble in water (e.g., lipids). An organic solvent can be particularly useful if homogenization of the food is part of the sample processing procedure. Trichlorotrifluoroethane (freon) has been the most common organic solvent used, but with the increased concern about environment effects of fluorocarbons, alternative solvents have been studied (Atmar et al., 1993, 1995; Le Guyader et al., 2004). Of these, chloroform : butanol (1 : 1, vol : vol) has performed well as an alternative (Le Guyader et al., 1996, 2000, 2004b; Dubois et al., 2002).

4.3. Virus Concentration

A variety of strategies have been used to concentrate viruses. In most instances, an elution step is used prior to the concentration step. An antibody capture assay using virus-specific antibodies attached to a fixed substrate was the first virus concentration procedure that allowed successful detection by

RT-PCR of HAV in shellfish implicated in an outbreak (Jansen et al., 1990). Since that time, additional antibody capture assays have been described using immune serum globulin and hyperimmune serum (Schwab et al., 1996; Gilpatrick et al., 2000; Kobayashi et al., 2004). Antibody is attached to a solid substrate such as paramagnetic beads, and after virus capture, repeated wash steps are used to remove substances that inhibit virus detection by molecular methods. A similar approach has been described using carbohydrate ligands for norovirus capture (Harrington et al., 2004). The potential utility of this approach is limited by the availability of broadly reactive antibodies to target viruses. For example, there is only one serotype of HAV, and antibody capture assays have worked well for this virus (Cromeans et al., 1997). This approach may not work with other enteric viruses that have multiple serotypes or antigenic types. The genetically and antigenically diverse noroviruses are an example: hyperimmune serum generated against recombinant viral protein only recognizes strains closely related to the one from which the recombinant protein was derived (Atmar and Estes, 2001). Although broadly cross-reactive monoclonal antibodies to noroviruses have been used to develop antigen ELISAs (Hale et al., 2000), the utility of these antibodies in antibody capture assays remains unproved.

Organic flocculation and polyethylene glycol (PEG) precipitation were originally used to concentrate viruses from shellfish for analysis in infectivity assays (Williams and Fout, 1992). In organic flocculation, virus is precipitated in the presence of beef extract by lowering the pH of the solution (to 3.0–4.5), and the pellet obtained after centrifugation is suspended in a small volume of sodium phosphate buffer (pH 9–9.5). No adjustments in pH are needed for the PEG precipitation. PEG-6000 was able to concentrate a variety of viruses (Lewis and Metcalf, 1988) and performed better than PEG-8000 in a direct comparison (Traore et al., 1998).

Ultracentrifugation is commonly used in research laboratories to concentrate viruses. The method has been used successfully to concentrate viruses extracted from shellfish prior to extraction of viral nucleic acids (Pina et al., 1998; Muniain-Mukija et al., 2000). The utility of the method is limited by the expense of an ultracentrifuge and the time required to pellet the virus (time of centrifugation increases as the *g*-force generated by centrifuge decreases). For these reasons, relatively few laboratories have used this approach for virus concentration from food samples.

4.4. Nucleic Acid Extraction

There are two main approaches that have been used to extract viral nucleic acids from concentrated samples: phenol: chloroform extraction and guanidinium isothiocyanate extraction. Enzymatic digestion (e.g., proteinase K) may be used as an initial step prior to phenol: choroform digestion. Several additional steps have been used to further clean-up the sample, including the use of organic solvents, suspended silica, affinity purification, and certain proprietary compounds (Jaykus, 2003). One of the more robust methods that can be adapted for nucleic acid extraction from a variety of substrates was orig-

inally described by Boom et al. (1990). The method uses chaotropic agent guanidinium thiocyanate to extract nucleic acids while inactivating nucleases. The nucleic acids are adsorbed onto a solid silica substrate following which impurities and inhibitors can be washed away before the nucleic acids are eluted and used for analysis.

When immunocapture methods are used for virus purification and concentration, the viral RNA can be released by heat denaturation into a small volume of buffer (Gilpatrick et al., 2000). Heat release is a rapid and simple method to use, but the nucleic acid must be analyzed the same day or it may be lost due to degradation by residual nucleases.

5.0. QUALITY CONTROL

Quality-control measures must be incorporated in any molecular diagnostic method. For example, the ability of PCR to detect as few as 10 copies of target sequence can lead to problems with cross-contamination between experiments or even within the same experiment and lead to false-positive results. On the other hand, failure to remove inhibitory substances during nucleic acid purification steps or the inefficient recovery of virus from a sample can lead to false-negative results. Steps (Table 5.5) to prevent or detect these problems should be routinely incorporated into any molecular detection procedure.

Table 5.5 Quality-Control Measures for Molecular Detection Assays

1. Prevention of cross-contamination
   - (a) Engineering controls
     - Separation of specimen intake, processing, set-up, and analysis areas
     - Use of dedicated equipment
     - Use of plugged pipette tips
     - Use of gloves, dedicated lab coats
     - Unidirectional workflow
     - Use of closed systems
   - (b) Experimental procedures
     - Uracil-*N*-glycolase
     - Photochemical inactivation: isopsoralen
2. Use of positive and negative controls
   - (a) Extraction controls
     - Known negative sample
     - Seeded positive sample
   - (b) Experimental control
     - Reagent control
     - Positive control
3. Inhibitor detection
   - (a) Internal standard
   - (b) Housekeeping genes

5.1. Prevention of Cross-Contamination

Cross-contamination of samples is one of the major problems encountered with the application of target amplification systems, especially with PCR assays. Millions to billions of copies of DNA amplicons per microliter are generated in each reaction. Thus, each nanoliter of reaction mix contains thousands to millions of copies of amplicons. Unrecognized microscopic contamination of equipment, clothes, and the environment can occur with daily laboratory activities and lead to false-positive results. Thus, it is important to take steps to prevent this problem and to identify the event if it occurs. The most experience in dealing with cross-contamination has been with PCR-based assays as described below.

A number of engineering controls should be used to prevent cross-contamination (Kwok and Higuchi, 1989). Ideally, the laboratory should have separate areas (rooms) for the following activities: specimen intake (cataloging and storage of samples to be analyzed), specimen processing (virus concentration and nucleic acid extraction), assay set-up, and postassay analytic assays. Workflow for an individual also should proceed in only one direction. Thus, a technician should not perform post-PCR analyses and then process new samples or set up new assays on the same day, unless she or he showers and changes clothes. Because aerosols can be generated during pipeting procedures, laboratories that perform large numbers of assays may incorporate measures to control airflow, with postanalysis areas being negative pressure in relation to other parts of the laboratory and preassay areas being positive pressure.

The work area should be cleaned with sodium hypochlorite solution to remove nucleic acids at the beginning and end of each work day and at any other time when there is concern that contamination of the work space may have occurred. Dedicated laboratory coats should be worn in only one area, gloves should be worn and changed frequently, and laboratory equipment should be dedicated to a single work area. The use of plugged pipette tips or positive displacement pipetors can help prevent contamination of equipment. Contamination of the work area with amplicons can also be prevented through the use of closed assay systems (e.g., as with real-time PCR assays). The sample to be analyzed is added to an analysis device that is then sealed and not opened again. Thus, there is no opportunity for the nucleic acids generated in the amplification reaction to contaminate the laboratory.

Additional control measures can be used to inactivate nucleic acids that may escape the engineering controls described above. Uracil-*N*-glycosylase (UNG) is a DNA enzyme that removes uracil residues from DNA molecules (Pang et al., 1992). If dUTP is used in place of dTTP in the reaction mix, the generated amplicons will contain uracil in place of thymidine. Treatment with UNG will degrade contaminating amplified DNA amplicons that contain uracil residues but not the thymidine-containing DNA (e.g., from native DNA). Because UNG has no activity against RNA molecules, it would not be effective in NASBA assay systems. UNG is added to the sample at the

beginning of the reaction and is then heat inactivated during the initial heat denaturation. Potential problems that may be encountered when using UNG include the following: decreased amplification efficiency due to less efficient incorporation of uracil into DNA, need to re-optimize amplification reaction conditions when replacing dTTP with dUTP, inability to have primer annealing temperatures below 55°C (due to residual enzymatic activity that could degrade newly formed amplicons), and inefficient degradation of short amplicons (Espy et al., 1993; Nolte and Caliendo, 2003).

Another approach that can be used to control DNA carryover contamination in PCR assays is the addition of isopsoralen to the reaction mix. After amplification, and before opening the reaction vessel, the sample is exposed to long-wave ultraviolet light, which cross-links isopsoralen to the DNA and renders it resistant to further amplification. Although this approach is simple, it is inefficient at inactivating short (<100 bp) amplicons. In addition, cross-linking may interfere with hybridization and isopsoralen may inhibit amplification (Nolte and Caliendo, 2003).

5.2. Use of Positive and Negative Controls

Several controls should be incorporated into each assay. Negative controls should include an extraction control and a reagent control. The use of an extraction control (a known negative sample) can allow the identification of cross-contamination during the virus concentration and nucleic acid extraction processes. A negative reagent control allows the identification of contamination of one or more of the reagents used to perform the assay. Some investigators choose to use more than one extraction or reagent control in an assay. Although this approach is more likely to detect the low-frequency environmental contamination events (e.g., contamination of a reaction tube with an aerosol containing amplicons), it increases the costs of performing the assay.

Positive controls should also be incorporated into each assay. An extraction positive control can demonstrate the efficiency of a concentration/extraction procedure. The other positive control (purified nucleic acids added to the reaction mix) will demonstrate that all of the necessary reagents are added to the reaction mix and that all enzymes are functional. Care must be taken not to allow the positive control to cross-contaminate other samples. It is prudent to use the smallest amount of positive control necessary to consistently obtain a positive result.

5.3. Inhibitor Detection

Even after extensive sample processing, inhibitors of reverse transcription or of PCR may remain. A variety of biologic and chemical substances that are present in foods or are used during sample processing have been found to act as inhibitors, including polysaccharides, heme, phenol, and cations. These inhibitors can affect the results obtained with both qualitative and quantitative assays (Abu Al-Soud and Radstrom, 1998). Thus, it is important to minimize the presence of inhibitors that may lead to a false negative.

Several approaches are used to detect inhibitors in a sample. A housekeeping gene that is copurified with the target nucleic acid is often used as a second target to demonstrate that amplification can occur. However, this approach has not been used for foods because of the problem of identifying an appropriate housekeeping gene. More commonly, a standard control is added to the sample. The control may be nucleic acids that are amplified and detected by the virus-specific primers and are amplified in the same reaction vessel or they may be added to a separate reaction vessel and be amplified by a different set of primers. A disadvantage of adding the internal standard directly to the reaction mix is that amplification of the standard may competitively inhibit amplification of target viral nucleic acids. On the other hand, the use of different primers to amplify a standard added to a separate reaction vessel may fail to detect inhibition if the virus amplification assay is more easily inhibited than the assay for the standard. Assay-specific internal standards have been described for noroviruses, hepatitis A virus, and rotavirus (Atmar et al., 1995; Le Guyader, 2000; Parshionikar et al., 2004).

6.0. RESULT INTERPRETATION

Several factors must be considered in evaluating the results of molecular assays described above. First, all control samples must be examined. If negative control samples (extraction controls, reagent controls) are positive, then any other positive samples in the assay are suspect due to the possibility that cross-contamination has occurred. If a positive control sample turns out to be negative, it may mean inefficient processing (positive extraction control), inhibited enzymatic activity (internal standard control), or failure to add (or inadequate activity of) one of the reagents (e.g., enzymes, oligonucleotide primers, buffers).

Even if all controls work properly, it should be recognized that additional factors may affect the interpretation of the results. The sensitivity of the assay may be insufficient to detect virus that is present in the sample at a level below the limit of detection. A positive result indicates that the viral nucleic acid is present but it does not address the viability of the virus; nucleic acids can be detected using molecular assays even after the virus is no longer viable (Nuanualsuwan and Cliver, 2002). This observation has raised concern about the utility of molecular methods in the assessment of food safety (Richards, 1999). The importance of contamination of food with nonviable viral nucleic acids needs to be determined.

7.0. APPLICATION TO FOODS

Much of the work and early success in the development and application of molecular methods to foodstuffs have involved shellfish. Methods used to partially purify and concentrate cultivable enteric viruses were developed in

the 1970s and 1980s (Lewis and Metcalf, 1988). These methods were modified to allow detection of these and noncultivable viruses using hybridization and RT-PCR assays (Zhou et al., 1991; Atmar et al, 1993, 1995; Lees et al., 1994, 1995). As RT-PCR methods were developed, they were applied to shellfish samples implicated in outbreaks of HAV (Jansen et al., 1990) and norovirus (Le Guyader et al., 1996; Sugieda et al., 1996; Shieh et al., 1999; Prato et al., 2004). In some cases, sequence information obtained from infected persons was used to select primers to be used for assaying the shellfish (Le Guyader et al., 1996; Prato et al., 2004). These assays have also been used to identify virus contamination in shellfish in different geographic areas and during different seasons (Le Guyader et al., 2000; Formiga-Cruz et al., 2002) and to assess the level and duration of contamination in shellfish implicated in an outbreak (Le Guyader et al., 2003).

Methods to detect viruses in other types of foods have also been developed. These include 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; Jean et al., 2004; Le Guyader et al., 2004b). A limited number of studies have successfully applied these methods to detect viruses in foods associated with outbreaks of human disease. Schwab et al. (2000) detected a norovirus strain on contaminated ham associated with a cafeteria-associated outbreak, Le Guyader et al. (2004a) identified a norovirus strain in contaminated raspberries implicated in an outbreak, and Calder et al. (2003) identified hepatitis A virus in blueberries associated with a hepatitis A outbreak. These studies demonstrate that the tools to evaluate foods implicated epidemiologically during outbreak investigations are increasingly available. Additional investigation is needed before these methods can be applied for the screening of foods for contamination with food-borne viruses.

8.0. SUMMARY

Molecular assays are now available for the detection of the enteric viruses most commonly associated with the transmission of food-borne illness. Some of these assays, such as RT-PCR and NASBA, are able to detect very small quantities of virus genome and are suitable tools for the identification of viruses in foods. The application of these assays to foods has been facilitated by the development of methods for the concentration and purification of viral nucleic acids, but inhibitory substances may persist in processed samples and can lead to false-negative results. Thus, it is important to use positive and negative controls in every analysis to allow appropriate interpretation of assay results. There are now several reports of the successful identification of the same virus identified as the cause of a food-borne outbreak in the food implicated by the epidemiologic investigation. Additional studies are needed to determine the role molecular assays will have in food-safety programs.

9.0. REFERENCES

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