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MINIREVIEW

Application of PCR-Based Methods To Assess the Infectivity of Enteric Viruses in Environmental Samples[▽]

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The advent of the PCR has greatly enhanced our ability to detect human enteric viral pathogens in the environment, including water, municipal wastes, sewage, food, air, and fomites (2, 3, 59, 69, 79). This is especially true for those viruses which do not grow in cell culture. Despite great sensitivity, PCR methods do have some serious limitations for environmental viral analysis, including small sample volumes, the presence of PCR-inhibitory substances, and an inability to differentiate between infective and noninfective viruses (66). The ability of PCR to assess infectivity would greatly enhance its application for the monitoring of water and food quality and for treatment processes (e.g., disinfection). This review focuses on approaches to overcome these limitations.

DETERMINATION OF VIRAL INFECTIVITY

Viral infectivity can be described as the capacity of viruses to enter the host cell and use cell resources to ultimately produce infectious viral particles (virions) (10). The virion of most enteric viruses is composed of two major components, the capsid and the genome (83). The protein capsid is involved in the interaction of the virus with the host cell surface and contains antigens specific to cell receptors used to gain entry into the cell. The capsid also has the function of protecting the viral genome from degradation by nucleases and abiotic stresses, such as humidity, pH, UV radiation, and temperature. Thus, an undamaged viral capsule is critical for the initiation of a successful infection.

In addition to the viral capsule, the replication and translation of the viral genome to viral proteins and enzymes are also important for the successful production of new viral particles (83). The properties of the genome vary among the different groups of enteric viruses, which include positive-stranded RNA viruses, double-stranded RNA viruses, and double-stranded DNA viruses. Therefore, each viral group has its own mechanism for translation and replication of genetic information. Only positive-stranded viruses can initiate an infection by means of intact naked viral RNA without the viral capsid. However, this is very difficult and inefficient; in the case of poliovirus only 1 naked positive strand of RNA in 10,000 can initiate an infection (78).

Standard methods for the detection of infectious viruses in water require the use of susceptible cell lines within which the viruses can propagate and produce cytopathic effects (CPE) observable under a light microscope (17). It is important to emphasize that even with cell culture the detection of infectious viruses in environmental samples is difficult. Each virus has different capabilities to propagate in any given cell line. For example, not all enteroviruses can propagate effectively in any one cell line (15); therefore, the use of multiple cell lines is required to detect all the enteroviruses that may be present in a sample (72). In addition, detection of infectious viruses in a sample will greatly depend on the assay conditions, i.e., duration of exposure to host cells, volume of inocula, age of the cells, and the presence of inhibitory or toxic substances.

The advantages and disadvantages of cell culture for viral detection are summarized in Table 1. One important limitation is that some viruses, such as norovirus, cannot be grown in conventional cell culture. Detection of norovirus in particular relies largely upon direct reverse transcription-PCR (RT-PCR) of environmental samples, which does not provide information on infectivity (22, 43). Addressing the infectivity of slow-growing or noncultivable viruses is essential to understanding their persistence in the environment, the efficacy of disinfection, and ultimately the estimation of the risk of transmission to susceptible human populations.

DETECTION OF VIRUSES BY DIRECT RT-PCR/PCR

PCR-based methods have been successfully used to monitor water and food products for viral contamination (3, 7, 8, 14, 20, 23, 46, 82). During PCR, a fragment of the viral genome is amplified using specific primers. For RNA viruses, RT of the viral RNA to a cDNA strand (cDNA) is necessary prior to the PCR (68). During reverse transcription, a primer is necessary for the reverse transcriptase (RNA-dependent DNA polymerase) to initiate the synthesis of a cDNA from the RNA. Three types of primers are commonly used: random primers, polythymine primers, and specific primers. Random primers are short single-stranded DNA fragments with all possible combinations of bases. They will work as short nonspecific primers, and by using them, the RT reaction will nonspecifically produce cDNAs from the RNA present in the assay mixture (1, 87,

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TABLE 1. Advantages and disadvantages of use of cell culture and PCR for detection of viruses from water

Method	Advantages	Disadvantages	Comments	References
Cell culture	Direct measure of infectivity; large sample volume (usually between 1 and 7 ml/flask using 25- and 225-cm^2 flasks, respectively)	Time required for detection (varies between viruses, usually between 4 and 30 days); toxicity to compounds from environment (false positives); does not detect nonculturable viruses or slow-growing viruses (noncytopathic); may require multiple cell lines	Detection by observable CPE; susceptibility between different types of viruses varies between cell lines	17, 23, 63, 67
Direct PCR (RT-PCR)	Specificity and sensitivity; can be used for nonculturable or noncytopathic viruses; requires less time for detection, 2–4 h	Does not determine infectivity; affected by inhibitory compounds found in the environment (false negatives); low sample volume (usually between 10 and 100 μl)	Detection based on specific amplification of target regions in viral genome; in RNA viruses, a reverse transcriptase step is needed before PCR amplification; sensitivity based on number of viral particles per reaction volume	1, 3, 23, 66, 77

90). Polythymine (T_{16}) primers are usually 16-base-long thymine primers that will hybridize with the polyadenine end of the mRNA, where the reverse transcriptase will specifically transcribe mRNA (34, 90), or will hybridize with the 5′ end of the viral genome in the case of positive-strand RNA viruses and transcribe the entire viral genome (74). The use of specific primers will transcribe only the targeted region of the viral genome. The reverse transcription step is not necessary for viruses whose genome is composed of DNA.

Specific sets of primers are designed for the detection of each particular virus. Conserved regions or genes found in the viral genome allow for designing of primer sets capable of hybridizing with multiple members of a particular viral family. For example, a region of the adenovirus genome that codes for the production of the capsid hexon protein can be used for the detection of human adenoviruses such as types 2, 40, and 41 (5). The 5′ noncoding region of the enterovirus genome is used for designing primers for the detection of poliovirus, coxsackievirus, and echovirus (1). Other regions in the genome of the virus tend to have more variability and are useful for typing viral isolates in epidemiological studies (91). The final PCR product is analyzed by agarose gel electrophoresis, in which the correct size of the PCR product can be determined. However, confirmation of the PCR product by sequencing or by hybridization with internal nucleotide probes is highly recommended.

Real-time quantitative PCR (qPCR) is a type of PCR used to semiquantitatively determine the amount of original target present in the sample (29, 40). During a qPCR assay, the product produced during each cycle is quantified in two ways: by using SYBR Green (nonspecific attachment to double-stranded DNA), or by using a fluorescent internal probe (specific hybridization) (52). In both cases, fluorescence is measured during each cycle, and when the amount of fluorescence exceeds the background level (threshold level), the sample is scored as positive. The number of cycles needed to reach the threshold level, commonly referred to as the cycle threshold value, correlates with the amount of target in the sample prior to amplification (40). Real-time PCR is an excellent tool for environmental virology and has been used successfully to determine the concentrations of viral genomes in the environment (21, 28, 39). However, it also shares most of the disadvantages prevalent with PCR.

Multiplex PCR, which utilizes multiple primer sets within a single PCR, can be used to simultaneously detect different groups of viruses. However, this multiple viral detection can be difficult to optimize because of the different annealing temperature requirements of different primer sets and because of the potentially different properties of the viral nucleic acids found between viral groups (23, 24). In some cases, further confirmation steps such as oligonucleotide hybridization are needed in order to confirm the specificity of the detection (24). Real-time PCR (qPCR) has been more successful for multiple viral detection, because it can analyze each target independently in the same assay by using specific internal probes binding to different fluorochromes that the real-time PCR equipment can analyze independently (12, 41, 44, 54, 89). In addition, the PCR products can be of a similar size, ensuring a similar amplification efficiency for each target.

The advantages and disadvantages of direct PCR for the detection of viruses in the environment are listed in Table 1. In the case of viruses that grow poorly in cell culture the detection by PCR or the integration with cell culture and PCR (i.e., ICC-PCR) drastically reduces the time needed for detection (64). The time commonly needed for the detection of enteroviruses in water can be between 5 and 14 days using cell culture, 5 days using integrated cell culture, and less than a day using direct real-time PCR.

Because viruses are normally present in very low concentrations in ground or surface waters, large volumes of water are usually tested. To overcome this problem, viruses in the water

are concentrated, hence reducing the overall volume that needs to be assayed. In the VIRADEL (virus absorption and elution) method, large volumes of water (100 to 1,000 liters) are passed through a charged filter, and the virus adsorbs to the filter matrix due to electrostatic interactions between the virus and the filter matrix. This is followed by virus elution from the filter and reconcentration to a final volume of 25 to 35 ml (61). In neutral pH waters, the virus is negatively charged and can be absorbed using an electropositive charged filter. The virus is usually eluted from the filter matrix using a slightly alkaline solution of beef extract. When beef extract is used for viral elution, the sample can be reconcentrated by protein flocculation at low pH. During flocculation, the pH of the eluted sample is lowered to 3.5, the beef extract produces a floc, which is pelleted by centrifugation, and the virus is resuspended in 25 to 35 ml of buffer at pH 7.5 (88).

Another disadvantage of direct PCR is the limited sample volume that can be assayed (66). Normally, the total volume of the PCR mixture is between 25 and 100 μl, which limits sample sizes that can be assayed to less than 20 μl. In the case of qPCR the volume of target commonly used is 2 μl. In a large-volume PCR, the sample volume can be increased to 100 μl (3). In contrast, with cell culture it is feasible to assay 30 to 50 ml. The equivalent volume of the original sample using PCR often represents less than 10 liters from the original sample processed. Because of the differences in sample volume and assay volume, the actual sensitivity of PCR can be less than the sensitivity of detecting viruses directly in cell culture (66). The VIRADEL method typically yields concentrates of 25 to 35 ml (1, 88), which can be further concentrated down to 100 μl by using microconcentrators (85). While this increases the sensitivity of PCR, inhibitory substances are also concentrated, which can negate the additional volume reduction.

Internal controls for real-time PCR have been developed to determine the presence of inhibitors in a sample and ensure that samples that result in a negative PCR were negative because of the absence of the targeted virus, and not because of PCR inhibition (35). The treatment of the sample with resins, chelators, or commercially available kits can also be used to aid in the removal of inhibitors, such as humic acids or metals (1). However, some virus is usually lost during any purification process, and simple dilution of the sample may yield the same result (35). Either way, there is usually a trade-off between sample purification and sensitivity of detection.

DETERMINATION OF VIRAL INFECTIVITY USING PCR

Damage to the viral capsid may result in the loss of its capacity to protect the viral genome and its ability to replicate in the host. The detection of an intact genome can be an indication that the virus capsid is still in good condition, protecting the genome from degradation. Determining the relationship between damage to the viral capsid and degradation of the viral genome can provide information that can be used to correlate the detection of the viral genome with the infectivity of the virus. Two different RT-PCR approaches have been used for determining viral infectivity. One approach involves determining the presence of an intact genome or amplifiable undamaged genome by direct RT-PCR (48, 51, 74, 75). The second uses coupling of the RT-PCR with a pre-PCR sample treatment that can determine the integrity of the viral capsid prior to extraction and purification of nucleic acid and subsequent enzymatic amplification (56, 57). For the enzymatic amplification of the nucleic acids by RT-PCR, it is necessary that the target region of the nucleic acid is undamaged, since damage of the target region may result in inhibition of the RT-PCR. The use of direct RT-PCR for determining viral infectivity has been described for both positive-strand RNA viruses (48, 74, 75) and single-strand DNA viruses (4), including the amplification of the 5′ nontranslated region (NTR) of the viral genome (RNA viruses only), and the analysis of a large portion of the viral genome. Pre-PCR sample treatments with the potential to discriminate between infectious viruses and noninfectious virus include protease-RNase sample pretreatment, immunocapture of the virus from the sample, and the use of cell attachment to remove viruses from the sample. Table 2 summarizes the RT-PCR methods that have been used to discriminate between infectious and noninfectious viruses. With the exception of immunocapture followed by PCR, the application of the other approaches for environmental samples has been very limited.

Targeting the 5′ NTR of the viral genome by PCR. The *Picornaviridae* family of viruses are positive-strand viruses, with a genome that serves as mRNA. They have similarities with the mRNA of eukaryotes, including an internal ribosomal entry site in the 5′ NTR and a polyadenine tail in the 3′ end. The secondary structures and sequences of the internal ribosomal entry site found in the 5′ NTR are necessary for translation (83). The 5′ NTR has been reported as the most easily degraded region of the genome of the hepatitis A virus (HAV) upon exposure to chlorine and chlorine dioxide (9, 48). The lack of amplification of the 5′ NTR is accompanied by the loss of viral infectivity in cell culture. The first 600 bases of the HAV genome containing the 5′ NTR are more sensitive to chlorine degradation than the rest of the genome (48). Simonet and Gantzer (75) also analyzed the kinetics of poliovirus genome degradation using a qPCR approach during exposure to chlorine dioxide and reached a similar conclusion.

Analyzing a long target region of the viral genome by RT-PCR. An intact viral genome is necessary for the virus to remain infectious, and therefore analysis of longer regions of the genome by RT-PCR can screen for damage in the genome that eventually will reduce its infectivity. For RNA viruses, the primer selection for reverse transcription determines the portion of the viral genome that is transcribed to cDNA. For example, a poly(T) primer can be used to transcribe the entire poliovirus genome (74). The reverse transcriptase polymerizes a cDNA strand from the RNA, and if the RNA is damaged, the enzyme detaches from the RNA and polymerization ceases. The further the target region for the PCR is from the primer used for reverse transcription, the more likely it is that damage to the RNA exists, which will result in inhibition of the target cDNA sequence needed for PCR. Simonet and Gantzer (75) used this principle to compare the size of the genome region analyzed with the capacity to detect changes in the RNA of poliovirus and coliphage MS-2 after UV irradiation. For the production of MS-2 cDNA by RT, they used a primer that hybridized with the 3′ noncoding region of the genome (75). They found that the larger the genome region analyzed, the more likely the nucleic acid damage could be detected. How-

TABLE 2. Modifications of PCR (RT-PCR) used to infer viral infectivity

PCR method	Approach	Description	Advantage(s)	Disadvantage(s)	Reference(s)
5′ NTR RT-PCR	Targeting the 5′ and 3′ NCR of the viral RNA	These regions are more susceptible to degradation, specifically to chlorine and chlorine dioxide, which target the secondary structure of this region	The 5′ NTR is the most sensitive region of the *Picornaviridae* genome to degradation	May not work in other viral families which may have a different genome organization	9, 48, 49
LTR RT-PCT	Analysis of a long target region (LTR) of viral genome during amplification	Has been found to correlate length of region amplified and sensitivity of the RT-PCR to detect damage in genome	Increases the sensitivity of PCR to detect damage in the viral genome; it has been combined successfully with 5′ NTR RT-PCR	May reduce the sensitivity of PCR detection	4, 48, 74, 75
Enzyme treatment pre-RT-PCR	Treatment with proteinase and RNase before RT-PCR	The proteinase degrades damaged capsid and then the RNase degrades viral RNA; intact viral capsid may not be degraded by proteinase and may protect the RNA against RNase	Practical and easy step to incorporate into the assay	Detection of capsid damage caused by thermal inactivation is temperature dependent	56, 57
Immunocapture PCR	Antibody capture of the virus for RT-PCR	Damage in the viral capsid may change the antigenic properties of the virus, and specific viral antigen-antibody complexes may not form	Useful for isolating viruses from large volumes of water and from contaminated samples	Ability to discriminate between infectious and noninfectious viruses depends on antigenic properties of the viral capsid	19, 30, 55, 57, 71
Viral cell attachment and PCR	Attachment of virus to cell monolayer and detection of attached viruses by PCR	Inactivated viruses do not attach because of capsid damage to the cell monolayer and therefore the assay yields a negative PCR result	Potentially a large number of applications	Further studies are needed	57

ever, they also observed that fragment size alone could not be solely used to judge RNA damage among different viruses, as MS-2 exhibited greater resistance to UV than poliovirus for a similar fragment size. They pointed out that other factors, such as compactness or secondary structure of the genome, are also likely involved.

If the 5′ NTR end of the poliovirus genome is used as the target for subsequent PCR, using the poly(T) primer for reverse transcription, most of the genome needs to be transcribed to cDNA in order to have a target for PCR amplification. For example, with this approach, a 6,980-bp region of the 7.5-kb poliovirus genome was analyzed and a 3.0-$\log_{10}$ reduction was found in the amplifiable genome after exposure of the virus to 5 mg/liter of chlorine dioxide for 15 min (74). By using a primer in the 5′ NTR for reverse transcription and PCR, a 145-base-long fragment was analyzed and only a 1-$\log_{10}$ reduction of the amplifiable genome was found (74). However, a reduction of 4.5 $\log_{10}$ in infectivity was observed after 3 min of exposure to 5 mg/liter of chlorine dioxide, which indicated that even when analyzing longer regions of the genome by PCR, reduction of poliovirus infectivity was underestimated (54).

A similar principle was used to demonstrate the degradation of parvovirus DNA after amotosalen and UV treatment (4). However, for analyzing damage in the single-strand DNA genome of parvovirus, two PCR steps are necessary: the first PCR step involves the amplification of a long fragment of the viral genome, and the second step involves the use of qPCR to target a small portion of the fragment previously amplified. This approach results in a good correlation between an amplifiable viral genome and infectivity (4).

Treatment with proteases and nucleases before PCR. As mentioned previously, one function of the viral capsid is to protect the nucleic acid from degradation by nucleases found in the environment. The degradation of the viral capsid by protease will eventually expose the viral nucleic acid to nucleases. Nuanualsuwan and Cliver (56) used a protease and RNase pretreatment to differentiate between an intact virus and a virus inactivated by disinfection. The authors hypothesized that an intact viral capsid was less susceptible to protease degradation than a damaged capsid. The protease pretreatment degrades the capsid damaged by disinfection, allowing the nuclease pretreatment to degrade the unprotected nucleic

acid, yielding a negative PCR result. In contrast, an intact capsid protects the viral nucleic acid from nucleases and a positive PCR will result. Thus, the efficacy of the disinfection process can be assessed. This approach has been successfully used to determine the effectiveness of UV light disinfection, chlorine disinfection, and thermal treatment at 72°C in the inactivation of hepatitis A virus, poliovirus 1, and feline calicivirus (56). However, a drawback may be the inability of the processes to assess any thermal inactivation which occurs during enzymatic pretreatment due to long exposure at 37°C (57).

Immunocapture, cell receptors, and PCR. During infection, specific antibodies are produced against antigens on the viral capsid (83). The antigenic properties of viruses can be used for the production of specific immunoglobulins. Immunoglobulins can potentially recognize the viral antigen and attach to it, forming an antigen-antibody complex. In clinical laboratories, immunoglobulins are commonly used for the detection of viruses via methods such as the enzyme-linked immunosorbent assay. Immunomagnetic separation has been commonly used for the concentration of enteric protozoan pathogens and viruses such as noroviruses and enteroviruses from water samples (19, 18, 30, 71). In this technique, antibodies are attached to a surface or a paramagnetic bead. The target pathogen attaches to the antibody, and the magnetic bead is removed from solution with a magnet. The target pathogen can then be released from the antibody and detected by PCR. The main advantage of this technique is that the concentration step is specific and inhibitors are not concentrated. The use of immunomagnetic separation for samples with high concentrations of humic acids has been particularly successful.

However, the use of immunocapture for the detection of infectious virus will depend on the antigenic properties of the viral capsid and antigenic epitopes. The attachment of the viral capsid to the cell receptor is the first step before infection begins and is dependent upon the conformation of proteins on the viral capsid, which is responsible for the interaction with the cell receptors (53, 60). Changes in the conformation of the viral protein will inhibit the interaction with the cell receptors, and the ability of the virus to attach to the cell will be lost (57). In cases in which an antigenic epitope is involved in cell attachment, the success of the immunocapture will be related to the infectivity of the virus, since changes in the antigenic epitope will affect both, i.e., the binding of the antibody to the virus and the binding of the virus to the cell (31, 62). The opposite results may occur if the antigenic epitope is not involved in cell attachment (31). For example, an antibody capture system specific for HAV was unable to differentiate between infectious viruses and viruses inactivated by UV, chorine, or heat treatment, but an immunocapture system for poliovirus was able to differentiate between infectious virus and inactivated virus (57). It has also been reported that UV inactivation does not change the antigenic properties of hepatitis A virus (92).

Recently, receptors involved in the attachment of coxsackie B virus and adenovirus receptor and cell antigens involved in the attachment of norovirus (histo-blood group antigens) have been isolated and studied (37, 38, 80). The analysis of a cell receptor binding to a magnetic bead and RT-PCR have been used to differentiate between infectious and chlorine-inactivated coxsackie B virus (16). Porcine histo-blood antigens containing gastric murcin attached to a magnetic bead have also been used for the concentration and detection of norovirus in stool samples (84). The use of cell receptors and antigens involved in the virus attachment to the host cell may overcome problems associated with the production of antibodies mentioned previously, and more work is needed to determine the efficiency of their use combined with RT-PCR for the detection of infectious viruses.

Determination of viral attachment to the host cell by PCR. Nuanualsuwan and Cliver (57) studied interference with virus attachment to cell monolayers as a way of assessing viral inactivation by UV, hypochlorite, and heat. They demonstrated that inactivated viruses do not attach to cell monolayers and can be easily removed by rinsing the monolayer after incubation with the virus, resulting in a subsequent negative PCR. In contrast, a positive PCR demonstrates the presence of attached and infectious virus. This approach was successfully used in cell culture with poliovirus type 1, hepatitis A virus, and feline calicivirus (57). It would be interesting to determine if the same results could be obtained in cell lines in which the viruses cannot propagate effectively. For example, an infectivity assay has recently been described for the propagation of noroviruses, but this assay requires a cell line that is not widely available and special cell culture techniques, hence making it difficult to use on a regular basis (81). The use of cell attachment and PCR may be a practical alternative for the analysis of disinfectant effectiveness in the inactivation of norovirus because it may not require the cell differentiation processes necessary for propagation.

DETECTION OF VIRUSES BY ICC-PCR

Cell culture combined with PCR (ICC-PCR) is an approach that has been used to overcome most of the disadvantages associated with both conventional cell culture and direct PCR assays (63). Detection relies on an initial biological amplification of viral nucleic acid, followed by amplification via PCR (65). Viruses are allowed to replicate in cell culture for short periods followed by PCR amplification, which dramatically reduces the time necessary for viral detection (63). The advantages, disadvantages, and approaches to ICC-PCR are summarized in Table 3. ICC-PCR also has the advantage of detecting viable viruses that do not produce CPE. The sensitivity obtained with ICC-PCR is comparable to that obtained in cell culture after a second passage in cell culture (11). ICC-PCR reduces the time needed for detection of infectious viruses. In addition, fewer problems are encountered with inhibitory compounds that may be contained in environmental concentrates (13).

The use of ICC-PCR has been described for the detection of enteroviruses (65), hepatitis A virus (42, 64), enteric adenovirus (46), and astrovirus (36). The integrated use of cell culture with PCR has demonstrated a wide distribution of infectious viruses in water sources, since it allows for the detection of non-CPE-producing enteric viruses (47, 64). Lee et al. (47) demonstrated the simultaneous detection of both enteroviruses and adenoviruses in the same cell line with this approach.

Detection of viral nucleic acid intermediates during infection. During infection, the viral genome is transcribed to mRNA or another intermediary in the host cell which is eventually used for synthesis of viral proteins or replication of the

TABLE 3. Advantages and disadvantages of ICC-PCR and modifications for detection of viruses from water

Approach	Description	Advantage(s)	Disadvantage(s)	Reference(s)
ICC-PCR	Virus is amplified in host cell assays and subsequently detected by PCR	Toxicity can be identified; faster results than cell culture; larger sample volume; can detect noncytopathic viruses; less susceptible to inhibition than direct PCR	Does not detect nonculturable viruses; in cases of high-titer samples, can detect the viral genome without any growth in the host cells; may require multiple cell lines	63, 65
ICC and strand-specific RT-PCR	Specifically detects viral negative strand in cells in the case of positive-stranded viruses	Indication of infectious viruses in high-titer samples	Less sensitive than ICC-PCR	42
ICC and mRNA RT-PCR	Specifically detects the mRNA of adenovirus in cells after infection	Indication of infectious viruses in high-titer samples; increase in sensitivity	Same as ICC-PCR	45

genome (83). These steps are essential for viral replication. The detection of these intermediaries in the host cell during infection is a clear indication that the virus is replicating in the host cell and that it is infectious.

In the detection of a positive-strand RNA virus, the primer used is complementary to the sequence of the negative strand; the negative strand is transcribed to cDNA and then amplified by PCR (42). During cell infection with a virus such as HAV, the positive strand is transcribed to a negative strand in the host cell. This negative strand is used to produce more positive strands, which are eventually packaged in the viral capsule or used as templates to produce more viral mRNA. Thus, detection of a viral negative-strand RNA initiated by a positive-strand virus is a clear indication of infection. Strand-specific RT-PCR has been used in clinical studies for the detection of infectious hepatitis C virus from biopsy samples (70) and to demonstrate the replication of enteroviruses in valvular tissues from patients with chronic rheumatic heart diseases (50). It has also has been applied to the detection of hepatitis A virus using ICC and RT-PCR (42).

Detection of HAV using ICC and strand-specific RT-PCR depends upon the negative strand being detected in the cell extract after a successful infection. The sensitivity of HAV detection using ICC and strand-specific RT-PCR is one infectious unit (IU)/ml per cell culture flask within 4 days of incubation (42).

The same principle is used for the detection of adenovirus via mRNA RT-PCR (45). During infection, the adenovirus DNA needs to be transcribed to mRNA and the mRNA subsequently translated to functional proteins (i.e., DNA polymerase) as well as nonfunctional (capsid) proteins. Since double-stranded DNA is a very stable molecule, it is possible to detect DNA from noninfectious virus without the propagation of the virus in the host cell if the sample analyzed has a high concentration of adenovirus. A false positive can occur when the concentration of inactivated virus in the sample exceeds 10^3 IU/ml. In the detection of adenovirus using a combination of cell culture and RT-PCR, the detection of mRNA of adenovirus is a clear indication of viral infection, because the viral mRNA is only detected in the host cell during the infection. Ko et al. used two sets of primers for the detection of adenoviruses 2 and 41 (45): one for the early gene EA1, and another set for a late hexon gene (capsid protein). They found that the sensitivity of the mRNA detection varied between serotypes. The sensitivity of the assay after 7 days of infection was 0.2 IU for adenovirus 2 using the mRNA of E1A gene and 0.1 IU for adenovirus 41 using the mRNA of the hexon gene. The authors also compared the impact of chlorine and UV light disinfection on detection by cell culture and PCR. The copy numbers of mRNA for the hexon gene in the cells reached 10^5 copies after 36 h of infection. The high ratio of viral mRNA to viral DNA during infection resulted in an increase in the sensitivity of the assay (45).

STABILITY OF THE VIRAL GENOME AND ITS RELATIONSHIP TO VIRAL INFECTIVITY IN WATER

Direct RT-PCR analysis of water samples has become common during the last decade (2, 13, 29, 59, 85). However, the detection of viral genomes by direct PCR may not be an indication of the risk of exposure to an infectious virus. Therefore, understanding the relationship between a viral genome and viral infectivity is essential for the interpretation of PCR results. Table 4 summarizes studies which have compared the detection of infectious viruses with the detection of viral genomes in various types of water.

The interpretation of PCR results with those obtained by cell culture in the detection of viruses in water is difficult, because the ratio of infectious viruses to viral particles is variable. In the case of rotavirus grown in the MA104 cell line, there may only be 1 infectious virus particle out of a total of 40,000 virus particles (93). In the case of adenovirus and the PLC/PRF/5 cell line, the ratio is in the range of 1:1,000 (32). In river waters, the ratio of genome per infectious poliovirus has been found to vary between 26 and 46 (76). This infectious viral particle/total particle ratio is largely dependent on the assay method and how long the virus has been passed in the particular cell line. Thus, viruses from direct clinical or environmental samples have a much higher ratio than those viruses that have been adapted to cell culture (65). For example, the detection of all the infectious enterovirus in water requires multiple cell lines (15, 33), but they can be detected with only one set

TABLE 4. Comparison of virus inactivation and degradation of viral genomes in different sources of water

Type of water[a]	Log_{10} reduction in cell culture titer[b]	Viral genome detection (log_{10} reduction in genome concn)[b]	Comment	Reference
Surface water	4.5 in 14 days	3.0 in 14 days	Poliovirus	76
	1 in 5 days	1 in 7 days	Poliovirus at 25°C	6
	1 in 6 days	1 in 11 days	Murine norovirus at 25°C	6
	ND[c]	1 in 13 days	Norovirus at 25°C	6
Ground water	1 in 11 days	1 in 20 days	Poliovirus at 25°C	6
	1 in 25 days	1 in 100 days	Murine norovirus at 25°C	6
	ND	1 in 100 days	Norovirus at 25°C	6
Unfiltered seawater	ND	Negative[d] after 2 days at 23°C	RNA genome poliovirus RT-PCR targeting 5′ NTR[e]	86
	4 in 18 days	Negative after 11 days	Poliovirus initial concn of 10^4 PFU/ml at 22°C	95
Filtered seawater	ND	Negative after 28 days at 23°C	RNA genome poliovirus RT-PCR targeting 5′ NTR	86
	4 in 30 days	Detected after 60 days	Poliovirus initial concn of 10^4 PFU/ml at 22°C	95
Bottled water	3.5 in 16 days at 35°C	2 after 145 days at 35°C	Poliovirus initial concn of 10^5 MPN[f]/ml	27
Wastewater	2.4	1 after 60 days at 25°C	Poliovirus	78
Phosphate buffer (pH 7.2)	1 in 15 days; not detected after 89 days)	1 in 30 days (not detected after 180 days)	Coxsackievirus B3, $\sim 10^3$ MPN/ml RT-PCR target 5′ NTR	26
Phosphate buffer (pH 7.2) + clay[g]	1 in 19 days; not detected after 94 days)	1 in 100 days (not detected after 417 days)		26

[a] The unfiltered seawater was nonsterile seawater and was used to determine the effect of microbial activity on viral survival. Otherwise as noted most of the water samples used were nonsterile. Filtered seawater was passed through a 0.22-μm-pore-size filter.

[b] The log reduction was calculated using the following formula: $\log_{10}$ reduction = $\log_{10}(N/N_0)$, where N is the number of viruses at time T and N_0 is the number of viruses at time zero.

[c] ND, the infectivity of the sample was not determined.

[d] The sample was negative by RT-PCR detection.

[e] The primers were designed for targeting the 5′ NTR of the genome.

[f] MPN, most probably number.

[g] The concentration of clay was 200 mg/liter of Na-montmorillonite.

of primers. Correlating the detection of viral genomes with infectious viruses is problematic if there are no infectious viruses in the sample, or if the viruses present in the sample cannot be detected because the virus does not grow in the cell culture system used (14, 25). In some cases the addition of ICC and RT-PCR to the analysis allowed for the detection of infectious virus in samples that were negative by direct PCR (13, 96).

Microorganisms normally present in fresh and seawater play an important role in the inactivation of viruses and the degradation of the viral genome (94). Naked viral RNA can be detected up to 10 times longer in sterile seawater than in nonsterile seawater (86). The presence of microorganisms can also affect the relationship of infectious virus detection versus the detection of the viral genome. The detection of poliovirus by PCR in unfiltered seawater was found to be similar to its detection by cell culture in unfiltered seawater, but detection of the viral genome by PCR took twice as long as detection by cell culture (95). This is because the nucleases released by bacteria or other microorganisms may cause RNA degradation after viral loss of infectivity in unfiltered seawater. When viral infectivity was lost in filtered seawater, the degradation of the viral genomes was reduced, and no relationship was observed between viral inactivation and genome detection by PCR.

In another study, poliovirus detection in wastewater after 60 days decreased by 99% using cell culture, but genome detection by PCR only decreased 90% (78). In treated wastewater, the detection of the enterovirus genome has not been correlated with isolation of infectious viruses (25). In phosphate buffer, the addition of clay decreases the inactivation rate of coxsackievirus B and degradation of the genome (26). These results may explain the longer survival of enterovirus in wastewater because viral particles tend to attach to solids found in wastewater, reducing the inactivation rate of the virus.

DEGRADATION OF THE VIRAL GENOME AND ITS RELATIONSHIP TO VIRAL INFECTIVITY DURING DISINFECTION

The degradation of the viral genome by disinfectants can be estimated by determining the concentration or presence of amplifiable genome before and after exposure to a disinfectant. As described previously the sensitivity of this approach

TABLE 5. Degradation of viral genome after treatment with disinfectants

Disinfectant	Concn of disinfectant	Exposure	Reduction in infectivity (log_{10} reduction)[a]	Reduction on amplifiable genome (log_{10} reduction)	Virus	Reference
Chlorine	2,500 mg/liter	20 min at 24°C	Noninfectious	ND[b]	Rotavirus[c]	37
	1 mg/liter	6 min	4	3	Poliovirus 5′ NTR[d] 149 bases	51
	10 mg/liter	30 min	Noninfectious	ND	HAV 5′ NTR 1,023 bases[e]	48
Chlorine dioxide	5 mg/liter	25 min	ND[g]	3.5	Poliovirus (5′ NTR 6,989-base fragment)[f]	74
					Poliovirus (5′ NTR 169-base fragment)[g]	
	5 mg/liter	120 min	ND	4	HAV 5′ NTR, 1,023 bases[h]	49
	7.5 mg/liter	10 min	Noninfectious	ND		49
Ozone	0.37 mg/liter	10 s		3	Norovirus	73
		300 s		4.5		
	0.37 mg/liter	10 s	7	3	Poliovirus	73
		300 s		5		
Hydrogen peroxide	6%	20 min at 24°C	Noninfectious	ND	Rotavirus	58
UV light		200 μW/cm^2 for 2.5 h at 24°C	Noninfectious	ND	Rotavirus	58
		20 mJ s/cm^2	3	One	Poliovirus (1,869-base fragment)	75
		150 mJ s/cm^2	7	0.5	Poliovirus (78-base fragment)	75
		22 mJ s/cm^2	5	One	Poliovirus (5′ NTR 149 bases)	51
Ethanol	80%	20 min	Noninfectious	Detected[i]	Rotavirus	58

[a] The log reduction was calculated using the following formula: log_{10} reduction = log_{10} (N/N_0), where N is the number of viruses at time T and N_0 is the number of viruses at time zero.
[b] ND, the infectivity of the virus was not determined.
[c] The stock concentration of rotavirus was 10^8 PFU/ml.
[d] The primers were designed for hybridization with the 5′ NTR of the viral genome.
[e] The initial concentration of HAV was $10^{5.75}$ 50% tissue culture infective doses/ml. This RT-PCR analyzed almost the complete genome of poliovirus, and the detection limit of the reaction allowed only for determination of a ≥3.5-log reduction.
[f] The detection limit of this reaction allows for the determination of a 4-log reduction.
[g] The initial concentration of HAV was $10^{5.47}$ 50% tissue culture infective doses/ml.
[h] The rotavirus genome was detected, which means that the effect of ethanol did not inhibit the RT-PCR.

depends on the location and size of the fragment analyzed and is limited to the specific mode of action of the disinfectant evaluated (9, 48, 58, 74). Ma et al. (51) studied the relationship of PCR and cell culture after exposure of poliovirus to different disinfectants. They found that PCR results were comparable to cell culture results when assessing the disinfection ability of high levels of chlorine and high pH, because these conditions degrade the nucleic acids. Other disinfectants such as ethanol do not result in nucleic acid degradation, suggesting that PCR techniques may not be useful for the assessment of infectivity for agents or temperatures that do not degrade the nucleic acid of the virus (51). For example, the RNA of rotavirus remains amplifiable by RT-PCR after exposure to ethanol and drying, but not after loss of cell culture infectivity by chlorine and peroxide (58). Table 5 is a summary of the various studies in which the impact of disinfectants on detection of virus by PCR and cell culture have been compared. It has been reported that qPCR estimation of amplifiable genome treated with these disinfectants can be correlated with viral inactivation; however, this results in an underestimation of the inactivation rates compared to infectivity assays (48, 73, 74).

CONCLUSIONS

Several different approaches to assess viral infectivity using PCR have been attempted. PCR approaches that analyze damages to the nucleic acid that result in the impairment of the PCR include 5′ NTR PCR and analysis of the long regions of the viral genome. The use of these approaches may be restricted to positive-strand RNA viruses, such as enteroviruses and hepatitis A virus, because of the genome features of these viruses. Although it seems possible to use these approaches for studying norovirus, no work has yet been published. Another approach is to assess damage to the capsid, which results in loss of protection of the nucleic acid, or changes in the antigenic properties of the viral capsid to discriminate between infectious and noninfectious viruses. In this approach, enzyme pretreatment and assessment of viral attachment to cell receptors

can be used. Because the process of viral replication in the host cell varies with viral type, it is doubtful that any direct PCR method would be totally satisfactory for assessing viral infectivity. However, the application of these approaches provides a more reliable understanding of the factors that contribute to viral inactivation.

Currently, the combination of PCR and cell culture offers the best approach to assess viral infectivity, including the detection of slow-growing viruses such as HAV. In addition, it has been successfully used for the detection of adenovirus, enterovirus, astrovirus, and reovirus from the environment. However, there are difficulties in obtaining a cell culture model for the detection of important waterborne pathogens such as norovirus, leaving the use of direct PCR as the most feasible technique.

Presently, the interpretation of PCR results in the detection of viruses in water and assessment of disinfectant efficacy should be on a case-by-case basis considering the type of water, mode of action of the disinfectant, and the type of virus. However, some problems associated with the detection of viruses by direct PCR from the environment may reduce the possibility of analyzing large regions of the viral genome. The combination of this approach with viral capture systems, such as use of antibodies or cell receptors to separate virions from the environmental matrix, may help reduce the effect of PCR-inhibitory compounds and provide a more feasible approach for further analysis of the viral genomes in environmental samples.

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