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Diagnosis of Noncultivatable Gastroenteritis Viruses, the Human Caliciviruses

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INTRODUCTION

Importance and Impact of Gastroenteritis

Acute gastroenteritis is one of the most common diseases of humans. In the United States, it is second only to acute viral respiratory disease as a cause of acute illness (61). Worldwide, more than 700 million cases of acute diarrheal disease are estimated to occur annually just in children under the age of 5 years (230). Gastroenteritis most commonly is manifested clinically as mild diarrhea, but more severe disease, ranging from upper gastrointestinal symptoms (nausea and vomiting) to profuse diarrhea leading to dehydration and death, may occur. The annual mortality associated with gastroenteritis has been estimated to be 3.5 to 5 million, with the majority of deaths occurring in developing countries (21, 97, 250).

Acute gastroenteritis is caused by a number of different agents, including bacteria, viruses, and parasites. Until recently, many cases were attributed to viruses because of the failure to identify a bacterial or parasitic pathogen. Although Reimann et al. (214) and Gordon et al. (77) suggested more than 50 years ago that viruses are a cause of diarrhea after

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TABLE 1. Causal relationship to diarrhea of enteric viruses[a].

Group	Viruses	Cultivation reported[b] (reference)
Causal relationship demonstrated	Rotaviruses	Yes (66)
	Human caliciviruses	No
	Astroviruses	Yes (155, 156)
	Enteric (group F) adenoviruses	Yes (147)
Candidate agents (etiologic relationship not yet determined)	Coronaviruses	Yes (215)
	Echovirus type 22	Yes (148)
	Picobirnaviruses, picotrirnaviruses	No (35, 96, 174)
	Pestiviruses	No (13, 257)
	Toroviruses	No (17, 18, 251)
Other agents[c] (causal relationship not demonstrated)	Non-group F adenoviruses	Yes (148)
	Coxsackie A and B viruses	Yes (148)
	Echoviruses	Yes (148)

[a] Viruses are listed by relative clinical significance. Data are modified from R. L. Atmar and M. K. Estes, Clin. Microbiol. Newsl: **19:**177–182, 1997 (9), with permission from Elsevier Science.
[b] Virus cultivated from human enteric sample.
[c] Present in stools of non-ill individuals with frequency similar to that seen in ill subjects.

inducing illness in volunteers with stool filtrates free of bacteria, it was only in 1972 that a virus (Norwalk virus) was definitively identified as a cause of acute gastroenteritis (139). Since that time, the number of viral agents associated with acute gastroenteritis has increased progressively.

Viral Causes of Gastroenteritis

Many different viruses have been found in the stools of persons with gastroenteritis. However, a causative role for each of these viruses has not been established. Criteria to define a virus as an etiologic agent of gastroenteritis include (i) identification of the virus more frequently in subjects with diarrhea than in controls, (ii) demonstration of an immune response to the specific agent, and (iii) demonstration that the beginning and end of the illness correspond to the onset and termination of virus shedding, respectively (148). Table 1 lists those viruses established as etiologic agents of gastroenteritis and other viruses found in stools that have not yet fulfilled the aforementioned criteria (9). Coronaviruses (34, 62), picobirnaviruses and picotrirnaviruses (35, 96, 174), pestiviruses (13, 257), and toroviruses (17, 18, 251) are candidate diarrheal agents because they are associated with diarrheal illness in animals and have been found in the stools of humans with gastroenteritis. However, these viruses have not fulfilled the criteria needed to establish them as diarrheal agents in humans (134, 148) and will not be considered further in this review. Non-group F adenoviruses and several enteroviruses (e.g., Coxsackie A and B viruses) are found in the stools of non-ill individuals with frequencies similar to those seen in ill individuals (148).

Of the viruses that have been shown definitively to be causes of acute gastroenteritis, only the human caliciviruses cannot be grown in cell culture. The study of rotaviruses, enteric adenoviruses, and astroviruses has been facilitated greatly by the ability to propagate these viruses in cell culture. The ability to cultivate these viruses has allowed the production of reagents for use in diagnostic studies, a better understanding of factors correlated with immunity to infection, and the elucidation of each virus's life cycle. Although human caliciviruses have defied numerous attempts to propagate them in cell culture to date, recent developments in their study by using molecular biology techniques have increased our understanding of this group of viruses. This article will review the new diagnostic assays that have resulted from these advances.

HISTORY OF HUMAN CALICIVIRUSES

Recognition

In 1968, an outbreak of acute gastroenteritis (termed winter vomiting disease) occurred among students and teachers in a school in Norwalk, Ohio (1). The primary attack rate was 50%, with a secondary attack rate of 32%. Illness was characterized by nausea and vomiting in >90% and diarrhea in 38% of affected individuals, and the duration of illness was usually 12 to 24 h. Subsequently, organism-free filtrates of stools collected from affected individuals induced similar illness in human volunteers, and different treatments of the inoculum suggested that the causative agent was a small (<36 nm), ether-resistant (nonenveloped), relatively heat-stable virus (24, 57, 58). Attempts to propagate the agent in cell culture and organ culture were unsuccessful (24, 57).

In 1972, Kapikian et al. (139) used immune electron microscopy (IEM) to identify 27-nm viral particles, the Norwalk agent, in a fecal filtrate used to induce illness in human volunteers. Virus particles were precipitated in an antigen-antibody reaction using convalescent-phase serum from a volunteer who became ill following inoculation with the fecal filtrate. Antigen-antibody complexes were then visualized with an electron microscope. The assay was modified to quantify the amount of antibody in serum and demonstrated that significantly more antibody was present in convalescent-phase serum than in acute-phase serum. Because of these data, Norwalk virus (NV) was proposed to be the etiologic agent of the Norwalk, Ohio, outbreak of gastroenteritis (30). Later studies demonstrated that other small, round-structured viruses (SRSVs) morphologically similar to NV were associated with outbreaks of gastroenteritis (7, 59, 238), but NV remained the prototype of these fecal viruses.

Morphologically typical caliciviruses were first recognized in stool samples by Madeley and Cosgrove in 1976 (176). These investigators found calicivirus particles in the fecal specimens

TABLE 2. Classification schemes for HuCVs

Classification method	Groups
Morphologic	Small round viruses, SRSVs, "typical" caliciviruses
Antigenic	
IEM	IEM types, SPIEM types
Reactivity with VLP-specific immune sera	Antigenic groups
Cross-protection in experimental human infection	Antigenic types, possibly serotypes
Genetic (based on sequence information)	Genera (NLVs, SLVs), further divided into genogroups, further divided into genetic clusters

of 10 children, but some of the children were asymptomatic, so that no conclusions as to the pathogenicity of the virus could be made. Later that year, Flewett and Davies (68) identified calicivirus particles in the small bowel from a fatal case of gastroenteritis, but because adenovirus particles also were present in large numbers, the significance of the calicivirus particles could not be determined. However, in the next several years, caliciviruses were definitively associated with several outbreaks of gastroenteritis (37, 38, 44, 45, 185) and became recognized as another virus group associated with gastroenteritis (33, 175).

Based on properties of NV and its appearance by electron microscopy, NV and other small round viruses were thought to be parvovirus-like (57, 137). However, in 1981 Greenberg et al. (93) published data suggesting that NV has a single structural protein with an estimated molecular mass of 59 kDa and proposed that Norwalk virus might be a calicivirus. Nine years later, Jiang et al. (216) provided molecular evidence that NV is a calicivirus by demonstrating that the viral genome consists of positive-sense, single-stranded, polyadenylated RNA. Subsequent elucidation of the complete sequence of NV and related SRSVs confirmed the genetic relatedness of these viruses to other caliciviruses (55, 108, 131, 153, 154, 225).

Classification

The classification of viruses responsible for acute gastroenteritis was first based on morphology (Table 2). For example, NV was the prototype of a group of agents initially called SRSVs. Recently, rapid advances in molecular biology allowed these viruses to be classified based on their genome characteristics, and most of the previously named SRSVs were shown to belong to the *Caliciviridae*.

Morphology. The virions are composed of a single major capsid protein. Structural analysis of virus particles from stool is limited by the small number of particles present in these samples. However, by negative-stain electron microscopy, the NV has an indistinct "feathery" outer edge and an indistinct surface substructure, although there is a suggestion of indentations on its surface (Fig. 1A). Expression of the capsid protein using the baculovirus system results in the self-assembly of the NV protein into virus-like particles (VLPs) (Fig. 1B). By negative-stain electron microscopy, these VLPs have a morphology similar to that of the native virus. The structure of these VLPs has been resolved by electron cryomicroscopy and computer image processing as well as by X-ray crystallographic methods (211, 212). The capsid exhibits a $T = 3$ icosahedral symmetry. The major structural protein folds into 90 dimers that form a shell domain from which arch-like capsomers protrude. A key characteristic of this architecture is 32 cup-shaped depressions at each of the icosahedral fivefold and threefold axes. These cup-like depressions are more prominent in some strains (particularly the Sapporo-like viruses [SLVs]), leading to the characteristic "Star of David" appearance from which caliciviruses get their name (Fig. 1C). The name calicivirus is derived from the Latin calyx, meaning cup or goblet, and refers to the cup-shaped depressions visible by electron microscopy.

Genomic organization and classification. In 1990, the genome of NV was cloned and characterized (124). More recent studies have characterized and completely sequenced this and other human viruses in the family (55, 108, 131, 153, 154, 170, 171, 225). Recently, the International Committee on the Taxonomy of Viruses established and approved four genera within the family *Caliciviridae*, including two human calicivirus (HuCV) genera (86). These nonenveloped viruses have a diameter of 27 to 40 nm by negative-stain electron microscopy and a buoyant density of 1.33 to 1.41 g/cm^3, and they contain a positive-sense polyadenylated single-stranded RNA of approximately 7.6 kb (Fig. 2). The two HuCV genera currently have the tentative names of "Norwalk-like viruses" (NLVs; type strain, NV [Hu/NLV/Norwalk virus/8FIIa/1968/US]) and the "Sapporo-like viruses" (SLVs; type strain Sapporo virus [Hu/SLV/Sapporo virus/1982/JA]). The proposed nomenclature for HuCVs is species infected/virus genus/virus name/strain designation/year of isolation/country of isolation. Table 3 shows the strain name and proper name for common NLV and SLV strains and for the strains discussed in this review. Common names will be used for the human virus strains throughout the rest of the review.

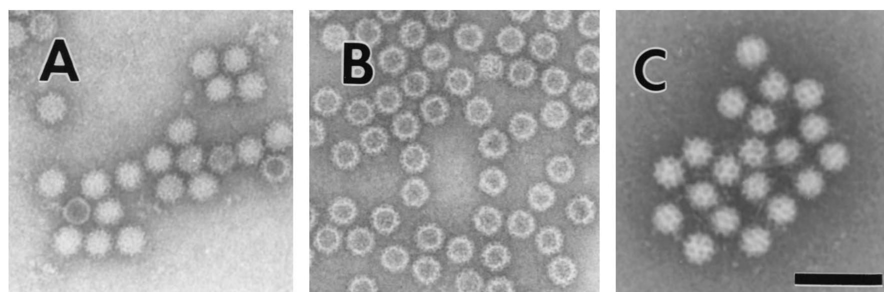

FIG. 1. Electron micrographs of (A) NV, (B) baculovirus-expressed NVL particles, and (C) Sapporo virus. Bar, 100 nm.

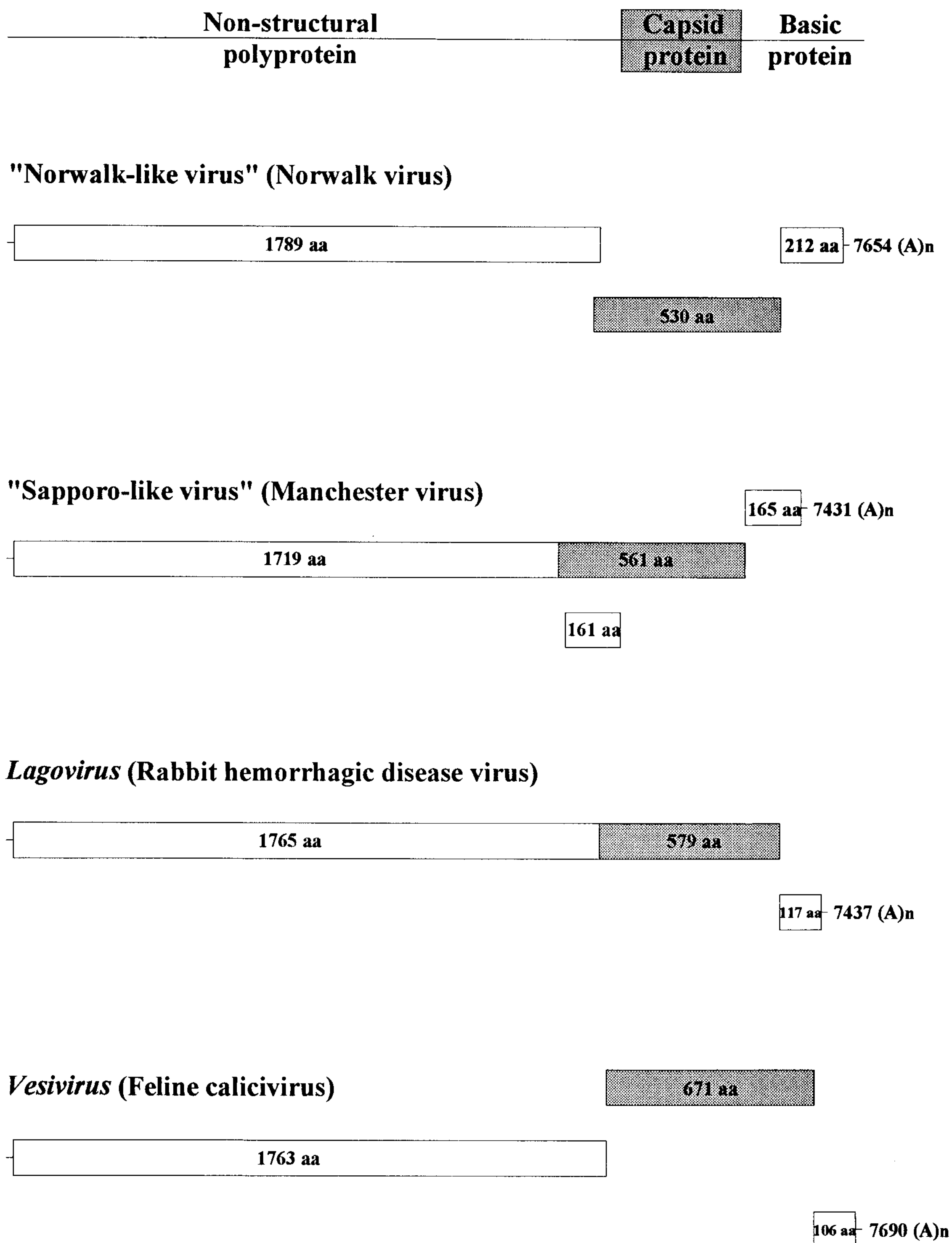

FIG. 2. Schematic of the genomic organization of viruses from the four different genera of the *Caliciviridae*. Strains [GenBank numbers] for which the entire genomic sequence is available are presented for each of the genera: NV [M87661] (108, 131), Manchester virus [X86560] (170, 171), rabbit hemorrhagic disease virus [M67473] (187), and feline calicivirus [M86379] (31). A nonstructural polyprotein (aa 1719 to 1789) is encoded by the 5′ end of the genome. The major capsid protein (shaded area) is in frame for the SLVs and lagoviruses, while it is in the +1 frame for NLVs and −1 frame for the vesiviruses. A basic protein is encoded at the 3′ end of the genome for all four genera. SLVs have another ORF (+1) overlapping the capsid ORF that is not seen in the other genera.

TABLE 3. Representative strains in the two HuCV genera[a]

Genus and genogroup	Virus common name (strain designation)	Cluster[a]
NLVs		
Genogroup I	Norwalk virus (Hu/NLV/NV/8fIIa/1968/US)	NV
	Southampton virus (Hu/NLV/SV/1991/UK)	SV
	Desert Shield virus (Hu/NLV/DSV395/1990/SR)	DSV
	Cruise ship virus (Hu/NLV/184–01388/1990/US)	CSV
Genogroup II	Snow Mountain agent (Hu/NLV/SMA/1976/US)	SMA
	Hawaii virus (Hu/NLV/HV/1971/US)	HV
	Mexico virus (Hu/NLV/MX/1989/MX)	TV
	Toronto virus (Hu/NLV/TV/TV24/1991/CN)	TV
	Lordsdale virus (Hu/NLV/LV/1993/UK)	LV
	Grimsby virus (Hu/NLV/GRV/1995/UK)	LV
	Gwynedd virus (Hu/NLV/GV/1993/UK)	GV
	White River virus (Hu/NLV/WRV/290-12275/1994/US)	WRV
SLVs	Sapporo virus (Hu/SLV/Sa/1982/JA)	Sa
	Manchester virus (Hu/SLV/Man/1993/UK)	Sa
	Parkville virus (Hu/SLV/Park/1994/US)	PV
	London virus (Hu/SLV/Lond/29845/1992/UK)	LoV

[a] Data from references 67, 86, 122, 195, and 196.

Calicivirus strains that infect animals (bovine and swine) and have characteristics that place them in the NLV and SLV genera have also been described (51, 100, 172, 233). In contrast, the other two genera within the family *Caliciviridae* (*Lagovirus* [type strain, rabbit hemorrhagic disease virus, Ra/LV/RHDV/V351/1987/CK] and *Vesivirus* [type strain, swine vesicular exanthema virus, Sw/VV/VESV/A48/1948/US]) are currently recognized to contain strains that naturally only infect animals (and not humans). There is a single case report of infection of a person who was isolating a vesivirus in the laboratory (229).

The genome of NLVs is organized in three major open reading frames (ORFs). For NV, the first ORF at the 5′ end encodes a large polyprotein of 1738 amino acids (aa) with a predicted molecular weight of 193.5 (193.5K). This polyprotein contains short motifs of similarity with the 2C (helicase), 3C (cysteine protease), and 3D (RNA-dependent RNA polymerase) proteins of picornaviruses. Thus, the 5′ end of the genome of the NLVs codes for a precursor of the nonstructural proteins. ORF2 encodes a 530-aa (56.6K) protein, the capsid protein. The ORF2 protein expressed in insect cells self-assembles into VLPs as explained below (Fig. 1B). ORF3 at the 3′ end of the genome is predicted to code for a small protein of 212 aa (22.5K) with a very basic charge (isoelectric point of 10.99). The ORF3 protein does not have sequence similarity with any other proteins in the GenBank database, and its function remains unknown. Recent studies indicate that the ORF3 protein is a minor structural protein, based on its being found in VLPs expressed from cDNA constructs that contain both ORF2 and ORF3 and in virus particles purified from stool (75).

The genome of SLVs is organized slightly differently. For Manchester virus, the first ORF codes for the nonstructural proteins as well as the capsid protein, which is found in-frame at the end of the nonstructural proteins (170, 171). This genome organization is similar to that found in the animal calicivirus rabbit hemorrhagic disease virus belonging to the genus *Lagovirus* (Fig. 2) (187). ORF2 encodes a predicted small, highly basic protein of unknown function, similar to ORF3 for NV. Manchester virus contains a third ORF within the capsid protein that could encode another small basic protein. The significance of this ORF is unclear, as this ORF is not seen in any of the other calicivirus genomes sequenced thus far, and the small protein it potentially encodes shows no sequence homology to other viral proteins in the database (171).

Sequence information has also been used to identify relationships between strains of NLVs and SLVs. Most comparisons have focused on the region of the genome encoding the viral RNA-dependent RNA polymerase or on the capsid protein gene (3, 5, 20, 43, 67, 109, 127, 161, 179, 180, 182, 197, 208, 243, 246, 255, 256), but comparisons of other genomic regions have also been made. Such comparisons have allowed the further subdivision of viruses in the NLV genus into two genogroups (Table 3) and viruses in both HuCV genera into clusters (6, 67, 122, 195, 196, 245) (Fig. 3). Table 3 identifies the genogroup and proposed genetic cluster for several reference strains. Figure 3 shows an unrooted phylogenetic tree of genogroup II viruses for which the entire sequence of the capsid gene is available, using the NV (genogroup I) capsid gene sequence as an outgroup. In most studies using the newly developed assays described below, these additional subdivisions have correlated with reactivity in the diagnostic assays, and they have been useful in evaluating virus transmission in molecular epidemiology studies (28, 50, 195, 245).

Cross-protection and serologic studies. Early volunteer studies examined the ability of different viruses to induce cross-protection. Based on these studies in addition to IEM, NV, Hawaii virus (HV), and Snow Mountain agent were defined as separate serotypes. Four distinct serotypes of the NLVs (NV, HV, SMA, and Hu/NLV/Taunton agent/1979/UK) and one serotype of the SLVs (Sapporo) were originally described by serologic IEM studies employing virus particles shed in stools

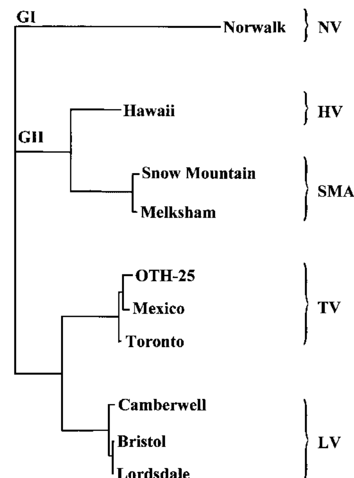

FIG. 3. Unrooted phylogram generated using the entire capsid amino acid sequence of nine genogroup II (GII) strains and one genogroup I (NV) strain and the PAUP program in the Genetics Computer Group suite of programs (73). Strains within different genogroup II genetic clusters are indicated, with the genogroup I strain NV serving as an outgroup. GenBank accession numbers used for this analysis are as follows: NV, M87661; HV, U07611; SMA, U75682; Melksham virus, X81879; Oth-25, L23830; MX, U22498; TV, U02030; Camberwell virus, U46500; Bristol virus, X76716; and LV, X86557.

as the antigen and paired sera from infected individuals as the source of antibody (133). Additional antigenic groups were proposed subsequently (165, 202). These serotype designations assumed that antibody reactivity by IEM reflects the reactivity of neutralizing antibodies. This may not be the case, and clear definition of serotypes remains difficult to achieve due to the lack of a cultivation system. More recently, the antigenic relationships between a subset of these viruses have been examined by enzyme-linked immunosorbentassay (ELISA) using hyperimmune antisera raised against VLPs. By this method, Norwalk, Mexico, and Grimsby viruses are antigenically distinct (102), as are NV and Desert Shield virus (161), NV and HV (87), and NV and Sapporo viruses (198).

FIRST-GENERATION DIAGNOSTIC TESTS USING HUMAN REAGENTS

During the 1970s and 1980s, tests for the diagnosis of NV infections were designed using reagents from previously infected humans. The number of virus particles in the stools of infected subjects is sufficiently small that hyperimmune sera could not be produced in animals. (An exception was the use of partially purified Sapporo virus to produce hyperimmune sera in guinea pigs [192].) The inability to propagate NV and related viruses in cell culture also prevented the production of animal hyperimmune sera. Thus, stools of acutely infected individuals served as a source of virus antigen, and convalescent-phase sera from infected individuals were used as hyperimmune sera. These restrictions limited the general availability of these diagnostic tools to only a few research laboratories (40). However, a number of tests were developed with these reagents and used to begin to define the epidemiology of NV and other human caliciviruses.

Electron Microscopy

Direct electron microscopy. Detection of enteric viruses in stool specimens using direct electron microscopy requires virus concentrations of at least 10^6 per ml of stool (56). In many laboratories, stool is mixed with phosphate-buffered saline or tissue culture medium to form a 10 to 20% suspension before being clarified by low-speed centrifugation. Subsequent concentration of virus may be achieved by ultracentrifugation or precipitation with ammonium sulfate. Virus particles are negatively stained using one of a number of available electron-dense stains, including phosphotungstic acid, uranyl acetate, and ammonium molybdate (56). Viral particles must be distinguished from nonviral material, and this can be particularly difficult for the NLVs that do not have typical calicivirus morphology (56, 140). The small numbers of viral particles present in fecal samples make direct electron microscopy, even after concentration, relatively insensitive. Nevertheless, direct electron microscopy is used to screen fecal specimens for enteric viruses in the public health laboratories of many countries, and it is clear that talented electron microscopists who receive fecal samples collected early in the course of an infection can often detect viruses (52, 179, 246, 256). However, this method requires highly skilled microscopists and expensive equipment, making it not feasible for large epidemiological or clinical studies.

IEM . The use of immune serum to aid in virus identification was first described for tobacco mosaic virus in 1941 (2). This method had been used for only a few human viruses (22, 49, 135) before Kapikian et al. (139) adapted it for the detection of NV in stool filtrates. Since then, it has been used to identify many other SRSVs in stool samples (27, 59, 141, 238, 242). A clarified stool suspension is incubated with a reference serum or saline (as a control) for 1 h at 37°C, and immune complexes are pelleted by ultracentrifugation (140). The pellet is suspended in a few drops of distilled water and negatively stained as for direct electron microscopy. The saline control is necessary to demonstrate the specificity of the immune serum because virus clumping may occur in the absence of antibody (56, 194). A positive sample has aggregates of viral particles which are absent in the saline control sample. Although IEM was first used to detect NV, it is positive on stool samples from only approximately half of volunteers who become ill following artificial challenge with NV (237). When IEM has been applied to outbreaks of gastroenteritis associated serologically with NV infection, it has been positive in only about 20% of the outbreaks, and just over one third of stool samples from affected individuals in these outbreaks are IEM positive (141). The lack of detection probably reflects the very low concentration of virus in many stool samples and the lack of collection of the first early diarrheal stool samples.

Modifications to the IEM method have been made to improve the ease with which viral particles are detected and to simplify the performance of the test. Solid-phase IEM (SPIEM) has been used to capture viral particles directly onto the grid (56, 164, 168). Virus-specific immunoglobulin or broad-spectrum immunoglobulin (gamma globulin) is used to coat the grids, and virus particles from a fecal suspension are then captured by interaction with antiviral antibodies. Protein A has been used to capture the antibody onto the grid before exposure to fecal suspensions; this may increase the exposure of antigen-binding sites by capturing the antibodies through their Fc receptor (56, 149, 165). SPIEM has been used as a tool for "serotyping" HuCVs (165, 166). Another modification of IEM has been the use of colloidal gold-protein A conjugates to label in suspension clumps of virus and antibody; this modification allows specific antigen-antibody interactions to be distinguished from nonspecific clumping (149).

IEM has also been used to detect seroresponses to viral antigen (37, 59, 139, 202). In this assay, the source of viral antigen is a stool filtrate in which viral particles are easily detected by electron microscopy or a stool from which virus has been partially purified. Antigen is mixed with a 1:5 dilution of the serum to be tested, and the mixture is examined. The amount of antibody is determined by the appearance of the viral particles and is rated 0 to 4+, with 0 being no viral aggregates noted and 4+ being nonglistening, heavily coated viral aggregates (139). This assay is type specific and has been used to evaluate the association of HuCVs with outbreaks of gastroenteritis (37, 56, 59, 202). While IEM is an important component of the diagnostic armamentarium for the noncultivatable caliciviruses, like direct electron microscopy, the application of IEM is limited and not readily applied to large epidemiological studies.

Immune Adherence Hemagglutination Assay

IEM was found to be a specific and reproducible method for antibody determination, but it is laborious, cumbersome, and time-consuming to perform. The immune adherence hemagglutmation assay (IAHA) was developed to allow the evaluation of Norwalk antibody levels in greater numbers of sera so that epidemiological studies of seroprevalence could be performed (138). Viral particles are purified from stool and used as antigen, and antigen-antibody-complement interactions are detected in a microtiter plate format by agglutination of sensitive human O erythrocytes. Kapikian et al. used the IAHA to demonstrate seroprevalence rates ranging from <20% in children to approximately 50% in adults in the fifth and sixth decades of life (91, 138). Although the assay has the advantage of requiring less antigen for its performance than complement fixation antibody assays, it was soon replaced with another assay, the blocking radioimmunoassay (RIA), which uses even less antigen and is more sensitive (91, 94, 136). IAHA also could not be adapted to detect viral antigen in stool specimens.

Radioimmunoassay

RIA was developed as an alternative to IEM for the detection of NV antigen in stool (91, 94). The assay is used in a microtiter format, and it detects both particulate and soluble antigen (133). Preinfection and convalescent sera from a volunteer experimentally infected with NV and known to have a high-titered antibody response (by IEM and IAHA) are used to capture virus antigen in duplicate wells. Immunoglobulin G (IgG) purified from a convalescent serum of a NV-infected chimpanzee or human volunteer and radiolabeled with ^{125}I is used as a detector system. The use of preinfection (negative) and convalescent (positive) serum differentially captures NV antigen and is indicated by greater radioactivity (counts per minute) in the convalescent than in the preinfection sample. Positive-negative (P/N) ratios greater than or equal to 2 indicate the presence of virus antigen in the sample (94). RIAs for the SMA and the morphologically typical HuCV were developed later (60, 191). In the latter assay, pre- and postvaccination sera from guinea pigs hyperimmunized with purified virus are used in place of human-derived reagents (191). For optimal specificity, positive samples must be confirmed using a blocking assay. Convalescent serum from a calicivirus-infected individual is incubated with captured virus antigen prior to addition of the radiolabeled IgG; a reduction in bound radioactivity of 50% or more indicates specific capture of virus antigen in samples with a P/N ratio of 2 or more (191). The RIAs using human-derived reagents do not require a blocking assay for confirmation of positive samples. All of these assays are specific (i.e., do not detect unrelated HuCVs or other enteric viruses) and 10- to 100-fold more sensitive than IEM (60, 94, 191). However, RIAs are negative when applied to stool samples from as many as one third of symptomatic volunteers experimentally infected with NV (231).

RIAs have been modified to detect virus-specific antibodies using a blocking format similar to that described above. A convalescent-phase serum from an HuCV-infected subject is used to capture partially purified virus antigen. Serial twofold dilutions of the serum to be tested for antibody determination are added next, and after an overnight incubation, ^{125}I-labeled, virus-specific IgG is added. If virus-specific antibodies are present in the serum being tested, binding of the radiolabeled IgG is blocked. A reduction in bound radioactivity of 50% or greater is used to define the presence of virus-specific antibodies, and the reciprocal of the last dilution at which 50% or greater blocking occurs is the titer of virus-specific antibody (94). RIA blocking assays have been developed for the detection of antibody to NLVs (NV and SMA and SLVs (60, 91, 94, 191). The RIA blocking assay for NV-specific antibody is 10 to >200 times more sensitive than the IAHA (91, 94).

RIA antigen and antibody detection assays were used to further characterize infection and illness in experimentally induced human infection (23, 48, 231), to perform seroprevalence studies in different populations (47), and to investigate outbreaks of gastroenteritis (14, 15, 46, 76, 92, 95, 98, 99, 141–144, 151, 169, 235, 253). The application of these assays helped identify NV and related viruses as a common cause of nonbacterial gastroenteritis outbreaks. For example, a review of 74 outbreaks of gastroenteritis investigated by the Centers for Disease Control between 1976 and 1980 showed that 42% of the outbreaks were associated with NLVs and an additional 23% were possibly associated with NLVs (141).

Enzyme Immunoassay

As the technology to perform immunoassays using nonisotopic reporters developed, enzyme immunoassays (EIAs) utilizing the same principles as the RIAs described above were developed for the detection of NV infection. Partially purified IgG is labeled with biotin or horseradish peroxidase in place of ^{125}I. The antigen detection EIAs detect NV in stool samples with a frequency similar to that seen with RIAs (72, 115, 178). Blocking EIAs for the detection of serum antibody are approximately twofold more sensitive than the comparable blocking RIA, although both assays detect fourfold or greater rises in serum antibody with similar frequency. An advantage of EIAs over RIAs is the increased stability of the reagents used to perform the tests. ^{125}I-labeled anti-NV IgG has a much shorter shelf life (several days to 2 weeks) than does anti-NV IgG labeled with biotin (3 months or more at −20°C) or horseradish peroxidase (6 months at 4°C) (72, 115). Other advantages of the EIA include the elimination of the use of radioisotopes and the decreased time needed to perform the blocking EIA compared to the blocking RIA (3 days and 6 days, respectively) (72).

EIAs for the detection of SMA and HV were developed later (178, 241), as were EIAs for the detection of IgM and IgA serum antibody responses (64, 65). The EIAs were applied in a fashion similar to RIAs, being used on specimens from experimental human infection studies (71, 177) and from outbreak investigations (16, 29, 114, 117, 249).

Western Blot Assay

Another approach used for the evaluation of antibody responses has been the Western blot assay. For this assay, virus was partially purified from stool for use as an antigen (112). Multiple bands appeared on blots used to assay human serum specimens, but when acute- and convalescent-phase sera were tested, increasing reactivity with a protein of approximately 63 kDa was identified. The specificity of this reactivity was con-

firmed using virus purified by isopycnic cesium chloride density gradient centrifugation (112). In some assays convalescent sera also showed increasing reactivity with a second band with an approximate molecular size of 33 kDa (201), which may represent the soluble protein identified following proteolytic cleavage of the capsid protein (111). When applied to clinical specimens from outbreaks of gastroenteritis, the Western blot assay gave results comparable to those obtained using IEM, although antibody detected by Western blot persisted for a longer period of time than did that detected by IEM (112, 145). The Western blot assay may detect a larger number of epitopes than IEM because it is able to detect antibody to related viruses that is not recognized by IEM (112). While this assay has theoretical advantages for the confirmation of HuCV infections, it has not been used by many laboratories, probably because of high background reactivities and lack of a reproducible source of viral antigen.

DEVELOPMENT AND APPLICATION OF NEWER DIAGNOSTIC TESTS

The successful cloning of NV led to the development of new reagents and methods for the diagnosis of infections caused by HuCVs. When the NV capsid protein was expressed in a baculovirus expression system, VLPs were generated (130). These VLPs were subsequently shown to be morphologically and antigenically similar to native virus particles (88). The VLPs were used to immunize different animal species to produce polyclonal and monoclonal immune sera that could then be used to establish EIA-based diagnostic assays. Virus sequence was used to design primer pairs for the detection of HuCVs using reverse transcription (RT)-PCR. The development and application of these newer assays are described below.

Antigen Detection

EIA with hyperimmune animal sera. The production of NV VLPs provided sufficient quantities of viral capsid antigen to allow the generation of hyperimmune sera in mice, guinea pigs, and rabbits (130). Hyperimmune sera from these animals have NV-specific antibody titers of 1:256,000 to >1:1,000,000. Subsequently, VLPs have been produced for other HuCVs, including Mexico virus (MX), SMA, HV, Desert Shield virus, Toronto virus (TV), Grimsby virus (GRV), Sapporo virus, Southampton virus, and Lordsdale virus (LV) (55, 87, 102, 109, 126, 160, 161, 198). Polyclonal hyperimmune animal sera produced by immunization of different animal species with VLPs have been used to develop antigen detection EIAs for use in clinical specimens (102, 121, 128). These immune sera have been quite specific, detecting homologous recombinant VLPs in an EIA format but not reacting with heterologous VLPs.

The antigen detection EIA utilizing polyclonal animal hyperimmune sera is set up in a sandwich format (79, 121). Rabbit hyperimmune serum is used for capture of viral antigen, and guinea pig hyperimmune serum is used to detect the captured antigen. The presence or absence of guinea pig serum is determined using a goat anti-guinea pig serum conjugated to horseradish peroxidase. EIAs using hyperimmune sera generated from recombinant NV or recombinant MX (rMX) VLPs detect similar amounts of antigen, identifying an estimated 10^5 to 10^6 VLP particles per well (79, 121). These antigen detection assays were found to detect native virus in stool samples with a sensitivity comparable to that of RT-PCR. The NV antigen detection assay was found to be more sensitive than RIA (79, 199). Of 50 EIA-positive stool samples obtained from experimental human infection studies, only 24 samples were positive by RIA; the 26 RIA-negative samples were shown to contain virus by RT-PCR. In contrast to the results of IEM studies in which viral shedding could not be documented 100 h following experimental human challenge, the antigen detection EIA identified NV in stool for up to 13 days (203, 237). Thus, the antigen detection EIA using hyperimmune sera raised against VLPs is more sensitive than earlier assays that relied on human reagents (88). The specificity of the assays for HuCVs has been shown by the lack of reactivity with other enteric viruses, including rotaviruses, adenoviruses, astroviruses, hepatitis A virus, and enteroviruses (79, 121, 199).

A limitation of these antigen detection assays was recognized when the assays were applied to clinical samples containing other HuCVs. For example, the antigen detection assay that utilizes hyperimmune sera raised to rNV VLPs only detects a subset of genogroup I NLVs and does not detect genogroup II NLVs (128, 161). Only the most closely related viruses in genogroup I (≥90% aa identity in the polymerase region) were detected in this assay. Similarly, the antigen detection assay that utilizes hyperimmune sera raised to rMX VLPs is most efficient at detecting genogroup II NLVs that are the most closely related to MX and does not detect genogroup I viruses and (104, 121). For example, an assay using hyperimmune sera raised to rMX VLPs does not detect the genogroup II NLV GRV in stool samples, and conversely, an assay using hyperimmune serum raised against rGRV VLPs does not detect MX in stool (102). These assays also do not detect SLVs. Thus, the lack of an EIA that is broadly reactive with a range of HuCVs has limited the utility of these assays. When applied to specimens in epidemiological studies (Table 4), positive stool samples have been identified infrequently with these assays in most studies (43, 118, 127, 193, 199, 213, 228, 254). In a few reports of outbreaks, antigen detection EIAs have been positive in more than 20% of samples tested (146, 167). When multiple assays have been used, results have been better, but it remains unclear how many individual assays will be needed to ensure the detection of most human caliciviruses. No EIAs using polyclonal sera are available yet commercially.

EIA with MAbs. Monoclonal antibodies (MAbs) have been prepared using native NV, native SMA, and rNV VLPs (110, 113, 239). Similar to what was seen with polyclonal sera, these MAbs are often type specific, recognizing the capsid protein of the immunizing virus but not that of other NLVs. The MAbs have been evaluated in limited studies for the detection of virus in stool samples. One assay format used a pool of two MAbs for antigen capture and also antigen detection. The detector antibodies were conjugated to horseradish peroxidase. This assay was reported to have a twofold greater sensitivity (as measured by the amount of virus detected) than assays using polyclonal sera, detected NV in 15 of 15 stool samples from subjects infected with NV, and failed to detect HV in any of nine stool samples from infected subjects (113).

In a separate study, a panel of 10 different MAbs were used for antigen capture, and antigen detection was performed using guinea pig anti-NV polyclonal antisera and horseradish

TABLE 4. Detection of HuCVs in stool specimens with antigen detection EIAs that use rVLPs as the antigen source for polyclonal hyperimmune antibody production

Study type and location	Infecting virus strain	VLP antigen	No. positive/no. of samples tested (% positive)	Reference
Experimental human infection				
U.S.	NV	rNV	35/41 (85)	79
		rMX	0/8 (0)	121
		rGRV	0/5 (0)	102
U.S.	SMA	rNV	0/8 (0)	121
		rMX	2/8 (25)	
U.S.	HV	rNV	0/8 (0)	121
		rMX	1/8 (13)	
Epidemiologic studies of sporadic cases of gastroenteritis				
Kenya		rNV	1/1,186 (<0.1)	193
		rMX	0/286 (0)	
India		rGRV	7/80 (8.8)	132
Japan/Southeast Asia		rNV	1/159 (0.6)	199
		rNV	2/155 (1.3)	118
		rMX	1/155 (0.6)	
Mexico		rNV	0/54 (0)	127
South Africa		rNV	0/1296 (0)	254
		rMX	9/1296 (0.7)	
		rNV	5/276 (1.8)	228
		rMX	12/275 (4.3)	
U.K.		rNV	0/187 (0)	43
		rNV	0/260 (0)	42
		rMX	1/260 (0.5)	
Venezuela		rNV	4/1120 (0.4)	213
Epidemiologic studies of outbreaks of gastroenteritis				
Japan/Southeast Asia		rNV	2/42 (4.8)	118
		rMX	3/42 (7.1)	
South Africa		rNV	1/3 (33)	255
		rMX	0/3 (0)	
U.K.		rMX	25/109 (23)	167
		rNV	0/unstated (0)	42
		rMX	15/192 (8)	
U.S.		rNV	5/19 (26)	146

peroxidase-conjugated goat anti-guinea pig immunoglobulin. All 10 MAbs tested in this study were able to capture NV (110). Subsequently, these MAbs were used in competition EIAs to map epitopes recognized on the capsid of rNV VLPs (107). Six to eight different epitopes covering five nonoverlapping regions of the capsid protein were identified. Three of the MAbs (NV3901, NV3912, and NV2461) recognized a single epitope and also captured Chiba virus VLPs, derived from a genogroup I virus with 75% amino acid identity to NV over the entire capsid. When NV3901 was used as the capture antibody in an antigen detection EIA with a polyclonal detector antibody, genogroup I viruses were detected in 9 of 15 fecal (RT-PCR positive) specimens, with positives representing four of five genogroup I genetic clusters (based on capsid sequence) assayed. The amino acid identities of the genogroup I viruses ranged from 63 to 70% over the entire capsid sequence compared to NV. The one genetic cluster not detected by the EIA had the lowest (63%) amino acid identity to NV (107). This is the first report of cross-reactive epitopes on NLVs. The failure to detect virus in all specimens by antigen EIA was thought to be due to differences in virus concentration in the samples tested, with RT-PCR having greater sensitivity than the antigen detection EIA, or to genetic variation among the genogroup I NLV genetic clusters (107). The description of a common epitope for genogroup I viruses leaves open the possibility that a similar common epitope may be present in genogroup II viruses. If all NLVs contain one or a limited number of common epitopes, the development of a broadly reactive antigen detection EIA will be possible. Such assays are desirable because large numbers of samples could be tested in a rapid and cost-effective manner. Alternatively, the genogroup I EIA may be combined with one or more less broadly reactive genogroup II EIAs to detect virus in stool samples. Such an approach, using an rGRV EIA for genogroup II viruses, compared favorably with results obtained by RT-PCR in an evaluation of specimens collected in southern India (132).

Antibody Detection

EIA with VLPs. The production of VLPs for NV and other NLVs has allowed the detection of immune responses to infection with these viruses. In these assays, VLPs are used to coat 96-well microtiter plates, and after blocking and washing steps, serial dilutions of human serum are added. Antibodies reacting with the VLPs are detected with goat anti-human immunoglobulin conjugated to an enzyme (e.g., horseradish peroxidase or alkaline phosphatase). The assay can detect total or class- or subclass-specific serum antibodies, depending on the reagents used to detect the bound human antibody (80, 116, 130, 240). The assay also has been modified to detect NV-specific IgA in fecal samples (203).

Two different approaches have been used to determine the amount of virus-specific antibodies in a serum specimen. The approach used by most laboratories is to perform a serial dilution of the serum specimen and to determine the last dilution that gives a reading above an empirically determined cutoff value (28, 41, 105, 116, 163). A second approach is to measure the amount of signal (e.g., optical density) from a single dilution of serum and to relate the measured signal to that measured using a standard reference serum (127, 189, 195, 207). Advantages of the latter method are that it allows a larger number of sera to be tested and is simpler and less expensive to perform. However, practical and theoretical problems limit the utility of testing a single dilution of serum. The practical problem is that well-characterized standard sera to be used as a reference reagent are not generally available, so that most laboratories cannot set up these assays. The theoretical problem is that the measurement of signal at a single dilution is affected by many factors besides the amount of antibody present in the sample, including the affinity of antibody in the sample for the test antigen and variability in the serum constituents that can affect antigen-antibody interactions. Thus, although antibody determinations using a single dilution of serum have been used in both seroprevalence studies (127, 207) and other epidemiological studies (195), most laboratories quantify virus-specific antibodies in serum using endpoint titration (28, 41, 105, 116, 163).

The VLP-based antibody detection EIA is more sensitive than assays using human reagents in RIA-, blocking EIA-, or IEM-based formats (88, 189). Total anti-NV antibody levels are 1.25- to >40-fold higher using an rNV VLP EIA assay than those obtained using RIA or blocking EIA. When applied to

sera from experimental human or chimpanzee infection and from outbreaks of gastroenteritis, the rNV VLP EIA detects fourfold or greater increases in serum antibody levels more frequently than IEM and at least as frequently as RIA and blocking EIA (88, 189). The antibody rNV VLP EIA identified infection following experimental human challenge better than the antigen detection EIA, detecting 40 of 41 (98%) infections compared to 36 of 41 (88%) infections (79). Assays utilizing other VLPs, including rMX, rTV, rHV, rSouthampton virus, and rLV, have also been developed (127, 195, 206).

The antibody detection EIA has been used to characterize IgG, IgM, and IgA serologic responses following experimental human infection with NV (80). Eight of 13 infected subjects had fourfold or greater increases in virus-specific antibody levels between 8 and 11 days following infection; ill subjects (eight of nine) were more likely to have these early responses, while antibody rises were seen in asymptomatic subjects only after 15 days. All infected subjects ($n = 14$) developed virus-specific IgM serum antibody. Virus-specific IgM serum antibody was present as early as 9 days following infection, but it did not develop in some subjects until 2 weeks after infection. IgM serum antibody could still be detected 3 months later in some subjects. Fourfold or greater increases in virus-specific IgA serum antibody were detected in all nine symptomatic infections but in only two of five asymptomatic infections. Virus-specific geometric mean serum antibody levels of infected and uninfected subjects were similar 3 months after challenge (80). The kinetics of the IgM and IgA responses are similar to those seen in earlier studies using human reagents (64, 65). The presence of neither virus-specific serum IgG, IgM, or IgA nor of fecal IgA is associated with protection from infection (80, 203, 240). Low serum antibody levels appear to be associated with a decreased likelihood of infection following experimental human challenge and natural exposure (79, 226).

Virus-specific IgM serum antibody has also been detected using an IgM capture assay. In this assay, goat anti-human IgM is used to coat microtiter plates, and dilutions of the test serum are then applied. VLPs are added in the next step, and VLPs bound by virus-specific IgM are detected using hyperimmune anti-NV rabbit serum (240). An alternative method uses a virus-specific MAb for VLP detection (28). Antibody levels are four- to eightfold higher using the IgM capture assay compared to the assay in which IgM bound to VLPs is detected (240). In two different studies following experimental human infection, IgM capture assays detected IgM responses in 15 of 15 and 14 of 15 subjects, with infection documented by fourfold or greater IgG responses (28, 240).

Detection of infection caused by heterotypic HuCVs. The serologic responses measured using VLP-based antibody detection EIAs have been characterized using sera collected during studies of experimental human infection and during evaluations of gastroenteritis outbreaks. Heterologous rNV IgG responses occur following experimental human infection with HV or SMA although they are present at a lower frequency and magnitude than is seen following infection with NV (240) or when rMX (SMA-like) VLPs are used in the assay (206). Heterologous rHV IgG responses also can be demonstrated following NV infection (41). The results are similar to those obtained using human reagents in blocking EIAs, although heterologous seroresponses as measured by the older and newer assays occurred in different subjects (177, 240). Heterologous seroresponses appear to be limited to subjects who also have an IgG seroresponse to homologous viral antigen and who are ill. Heterologous IgM and IgA responses occur infrequently (240).

Similar results have been obtained when these assays have been applied to sera collected during outbreak investigations (28, 105, 195, 206). IgG responses occur with a higher frequency and magnitude when the assay utilizes VLPs that are more closely related to the outbreak strain (105, 195). Noel et al. (195) found homologous IgG seroresponses to rNV VLPs when the infecting NLV was a genogroup I NLV with as much as 38.5% amino acid divergence from NV in the capsid region. In contrast, homologous seroresponses were seen for genogroup II NLVs only when the amino acid divergence from the test antigen was less than 6.5%. In general, the likelihood of detecting an IgG seroresponse for genogroup II NLVs was greatest when the test antigen was derived from a strain closely related to the infecting virus, less when the test antigen was derived from an unrelated genogroup II NLV, and least when the test antigen was rNV (genogroup I). Hale et al. (105) examined several outbreaks caused by genogroup II NLVs and found that rMX IgM responses occurred in 14 of 19 (74%) subjects with an IgG seroresponse. IgM responses occurred more frequently when the outbreak virus was MX-like (9 of 10) than when it was an unrelated genogroup II virus (4 of 9). Four of these subjects also had an rNV IgG seroresponse, but none of them had a rNV IgM seroresponse. Brinker et al. (28) obtained similar results applying the rNV and rMX IgM EIAs to genogroup I and genogroup II outbreaks. Twenty-four of 25 subjects infected with genogroup I viruses had rNV IgM responses, while only 3 of these subjects had rMX IgM responses; 28 of 47 subjects infected with genogroup II viruses had rMX IgM responses while none of them had rNV IgM responses. Taken together, these results indicate that IgM responses may be able to provide data on the genogroup of virus causing an infection.

The antibody detection EIAs have been used in seroprevalence surveys and in longitudinal studies of antibody acquisition (41, 54, 70, 82, 116, 118, 127, 163, 190, 193, 199, 204, 206, 207, 213, 227, 228, 236). These studies confirmed and extended the results obtained using human reagents showing that NLVs cause infection worldwide and seroprevalence increases with age. New findings include serologic evidence of NLV infection occurring in young children in developed countries that was not recognized in early studies (163) and of transplacental transfer of NLV-specific antibodies from mother to child (41, 118, 199, 206, 228). In addition, seroprevalence rates varied between regions within a country, between countries, and by the NLV VLP antigen used in the assay.

Nucleic Acid Detection

Nucleic acid detection assays are the third group of new assays that have been developed in the last decade since the cloning of the NV genome. Knowledge of the sequence of the NV genome led to the design of primers from the polymerase region that were able to amplify fragments of other NLVs and SLVs, and this led to sequencing of the complete genomes of many HuCVs. Although there have been a few reports of the

use of hybridization assays, the primary nucleic acid detection assay that is used is RT-PCR. RT-PCR is currently being used worldwide because of the lack of a commercially available, broadly reactive EIA. Both nucleic acid detection diagnostic approaches will be discussed below.

Hybridization assays. Only a few hybridization assays have been described for the detection of HuCVs. This is most likely due to the availability of the more sensitive RT-PCR assays at the time that cDNAs for HuCVs first became available. Jiang et al. (129) described a hybridization assay using ^{32}P-labeled cDNAs covering the same region of the genome as was amplified in RT-PCR assays. Stool suspensions (10 to 50%) were extracted with trichlorotrifluoroethane, and the viral nucleic acids were partially purified from the aqueous phase by digestion with proteinase K, extractions in phenol-chloroform and chloroform, and precipitation in ethanol. The hybridization assay detected NV with a sensitivity similar to that of RIA, but detected 100-fold less viral RNA and detected virus in stool samples 27% less frequently than an RT-PCR assay applied to the same samples. Thus, the hybridization assay was unable to detect NV when low titers of virus were present in a stool sample (129).

A hybridization assay using a digoxigenin-labeled cDNA probe derived from the polymerase region of Sapporo virus has also been described (150). Viral nucleic acids were partially purified as described above, and approximately 10^5 viral particles could be detected per dot. This level of detection is lower than the 1×10^4 to 2×10^4 particles detected by RIA and EIAs specific for Sapporo virus. The interpretation of test results was also hampered by the colorimetric detection system. Some stool samples gave false-positive results due to the color of the stool or to nonspecific binding of the probe to substances in the stool. The problem of false-positive results was addressed by inclusion of digoxigenin-labeled vector (pBR322) DNA in the assay as a negative control. The signal intensities obtained with virus-specific and control probes were compared to reference dots containing 10-fold serial dilutions of Sapporo virus cDNA, and positive samples were those in which the reaction with the virus-specific probe was stronger than with the control probe.

The Sapporo virus-specific dot blot assay was used to evaluate 100 stool samples in which HuCVs were detected by electron microscopy. Eight of 10 samples that tested positive using the SLV/Sapporo/82 EIA were positive by the dot blot assay, and an additional 13 samples were EIA negative and dot blot hybridization positive. Seventy-seven samples were negative by both assays. The investigators speculated that the improved sensitivity of the dot blot hybridization assay compared to the EIA may have been due to the greater conservation of sequence among SLVs in the polymerase region (targeted in the dot blot assay) than in the capsid region (targeted in the EIA assay) or to the presence of substances in stool samples that had a greater inhibitory effect on EIA than on dot blot hybridization assays. A potential advantage of the dot blot hybridization assay over RT-PCR assays is the lower cost of the assay and decreased risk of cross-contamination. Nevertheless, RT-PCR assays have been the major nucleic acid detection assay used for the diagnosis of HuCV infections.

RT-PCR. The first RT-PCR assays were described within 2 years of the initial report of the successful cloning of the NV genome (53, 129). Since then a number of different RT-PCR assay formats have been developed, and these assays have become one of the principal means for the diagnosis of HuCV infections. A number of factors can affect the sensitivity and specificity of RT-PCR assays, including the sample being assayed, the method used for purification of viral nucleic acids, the primers used in amplification, and the method used for interpretation of test results. Approaches to addressing each of these factors are discussed below.

TABLE 5. Extraction methods used to prepare a clinical sample for RT-PCR

Method	References
Antibody capture	74, 219
Chelation of multivalent cation impurities	103, 119
Exclusion chromatography	53, 103
Guanidinium-phenol-chloroform + alcohol precipitation	63, 232
GTC-silica	3, 101
Heat release	32, 221
PEG-CTAB	103, 129

(i) Extraction methods. Clinical samples frequently contain substances that can inhibit the enzymatic activity of the reverse transcriptase and DNA polymerase enzymes used in RT-PCR assays. Thus, it is usually necessary to partially purify viral nucleic acids or otherwise prepare the sample prior to the performance of the RT-PCR assay. Two major considerations in selecting an extraction method are its efficiency of viral nucleic acid recovery and its ability to remove or inactivate RT-PCR inhibitors. Secondary considerations include the ease of performance of the method and the number of samples that can be processed at one time. A number of different approaches for viral nucleic acid purification from stool samples have been reported. Those that have been used for the detection of HuCVs are shown in Table 5.

Jiang et al. (129) evaluated a number of different methods for the removal of inhibitors. Suspensions of stool samples (10 to 50%) were made, and the suspension was extracted with trichlorotrifluoroethane prior to further processing. Phenol-chloroform extraction, heating, and dialysis were all ineffective at removing the inhibitory substances present in stool samples, and oligo(dT)-cellulose chromatography of the sample was described as inefficient. The addition of cetyltrimethylammonium bromide (CTAB) after concentration by precipitation with polyethylene glycol (PEG) and proteinase digestion significantly improved the signal obtained following RT-PCR, and this method has been used in a number of subsequent RT-PCR studies of HuCVs (43, 127, 146, 159, 161, 188, 248, 255). Inhibitors can persist in some samples extracted with the PEG-CTAB method. Schwab et al. (221) and Hale et al. (103) found that approximately 13% (6 of 45) and 19% (7 of 36), respectively, of extracted samples still contained inhibitors that could prevent the detection of viral nucleic acids.

Modifications of the RNA extraction method of Chomczynski and Sacchi (39) have been used successfully to extract viral RNA from stool samples (63, 232). The method utilizes a mixture of guanidinium thiocyanate (GTC), phenol, and chloroform to extract the sample, followed by chloroform extraction and precipitation of nucleic acids in alcohol. The principal reagent is commercially available from a number of vendors (RNAzol, Ultraspec, and TRIzol). A variation on the GTC-

based extraction procedure was described by Boom et al. (26), in which a GTC-containing buffer is used to release viral RNA from the viral capsid. The viral RNA is adsorbed onto size-fractionated silica particles and washed in successive steps with a second GTC-containing buffer, 70% ethanol, and acetone. The viral RNA is then eluted from the silica particles with water. Other variations have also been used successfully (3, 101). The GTC-silica method has been reported to be quite successful in removing inhibitors of PCR in two comparative studies, performing better than the PEG-CTAB method in the detection of NLVs (103) and being approximately equivalent to PEG-CTAB for the detection of hepatitis A virus in stool samples (8).

Exclusion chromatography using spin columns containing Sephadex G200 was one of the original methods used to extract virus from stool samples (53). Although the method is sensitive, it is inconsistent at removing inhibitors from clinical samples (103). Similarly, a method based on the chelation of multivalent cation impurities has been used successfully for detection of NLVs but is unreliable at removing inhibitors of RT-PCR (8, 103). Unexpectedly, NLVs can be detected by RT-PCR in stool samples after simple heating of the sample to 95 to 99°C for 5 min (32, 221). The heat may inactivate some inhibitors and is thought to denature the viral capsid, allowing the release of viral RNA. Prior to the heat release procedure, 10 to 20% suspensions of the fecal samples are clarified and extracted with trichlorotrifluoroethane or pelleted through a 45% (wt/vol) sucrose cushion. The trichlorotrifluoroethane-treated samples must be diluted 100-fold, as amplification is inhibited in the majority of specimens at a lower dilution (221).

Antibody capture has been used for virus purification prior to amplification (74, 219). Virus-specific antibodies are bound in a 96-well plate or to paramagnetic beads before exposure to a virus-containing sample. After an incubation period to allow antigen capture, the wells or beads are washed repeatedly to remove inhibitors and other substances. Viral genomic RNA is then released from its capsid by heating. This method has worked well for hepatitis A virus, but its use in the detection of HuCVs has been limited. The principal reason this method has not been explored further is the lack of high-titered antisera that react with a broad range of NLVs. Nevertheless, Schwab et al. (219) were able to use human immunoglobulin preparations as a source of antibody to detect NLVs from water samples in which viruses had been concentrated by filtration and PEG precipitation. Polyclonal hyperimmune animal sera have also been coupled to paramagnetic beads and used to purify NV from fecal specimens prior to RT-PCR amplification (74). This strategy was found to yield a greater number of positive RT-PCR results from stools collected during experimental human infection studies than other processing protocols. As broadly reactive antisera become available, further studies of this extraction method are likely to be performed.

(ii) Primer selection. The sensitivity and specificity of RT-PCR assays depend in large part on primer selection. Several factors affect the ability of a primer pair to detect a given NLV or SLV strain, including primer sequence, the amount of virus present in the sample to be assayed, and the temperature used for primer annealing during the PCR amplification process. The genetic diversity of NLVs and SLVs has made it difficult to select a single primer set with adequate sensitivity and specificity to detect all NLVs. In general, regions of the genome with the greatest degree of conservation between strains within the genera and within genogroups have been targeted for amplification and primer design (Fig. 4A). But even within these regions, the nucleotide identity can be as little as 36% (2C helicase region) to 53% (3D polymerase region) between strains of different genogroups (5, 43, 182, 197, 245, 248, 255, 256). Within a genogroup of NLVs, greater conservation is seen, but the nucleotide identity between strains can still be as little as 60 to 64% (245). These observations have led to the design and use of primer sets targeting multiple areas of the viral genome (Fig. 4A, Table 6).

The majority of primers have been designed to amplify the most conserved region of the genome, the RNA-dependent RNA polymerase region (3, 20, 83, 125, 159, 180, 188, 220, 248). Although a number of primer pairs have been described, those described by Ando et al. (3), Green et al. (83), and Le Guyader et al. (159) have been the most frequently used (Fig. 4A, Table 6). Ando et al. (3) described a multiplex approach in which cDNA is made using a single primer (SR33) to initiate reverse transcription and four additional primers are used during the amplification process, three (SR48, SR50, and SR52) to amplify genogroup I NLVs and one (SR46) to amplify genogroup II viruses. Green et al. (83) described a different primer pair (E3 and NI) to amplify group II NLVs, and Le Guyader et al. (159) described a degenerate primer (NVp110) that can prime cDNA synthesis of group I and II NLVs and some SLVs. One of the problems associated with the genogroup II primer pairs (NVp110/NI and SR33/SR47) that amplify the polymerase region is that only 76 to 81 unique bases of sequence data can be obtained from the amplified products. Jiang et al. (125) recently described a primer pair, P289 and P290, that will amplify genogroup I and II NLVs and SLVs, yielding RT-PCR products from which almost 300 unique bases of sequence data can be derived. The P289 primer sequence is identical to the NVp110 and E3 primers in the 12 nucleotides at the 3′ end of the primer; the 5′ end has three nucleotide differences and is two bases longer than NVp110 and five bases longer than E3. These data suggest that NVp110, E3, and P289, whose 3′ ends are the reverse complement of the viral genome at the YGDD motif site of the RNA-dependent RNA polymerase, can successfully prime cDNA synthesis for a large number of NLVs and SLVs. Occasionally, primers designed based on the sequence of a locally circulating strain have performed better than other primer pairs (217). Other primers have also been described for the specific amplification of SLVs (20, 180, 244, 256).

Other regions of the viral genome have been targets of amplification (Table 6, Fig. 4A), including the 2C helicase, the capsid region, and ORF3 (53, 89, 101, 181, 188, 195, 248). The most common reason to target another area of the genome is to generate additional sequence data that might be useful in distinguishing or identifying unique viral strains. Toward this end, a RT-PCR assay that amplifies the 3′ end of the viral genome has been described (4). In general, assays using primers to amplify nonpolymerase regions of the viral genome are less broadly reactive (amplify a smaller number of virus strains due to the greater genetic diversity in these regions) or require a greater amount of virus to be present in the sample (e.g., to amplify ~3 kb of virus genome).

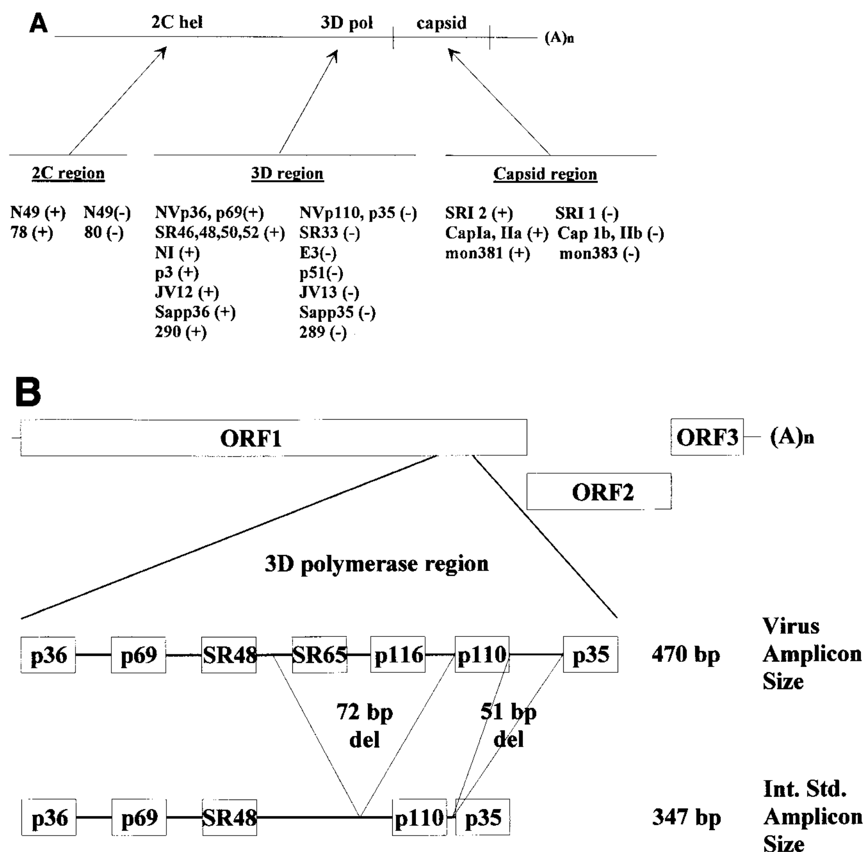

FIG. 4. (A) Schematic representation of the calicivirus genome and the regions amplified by common primer pairs. Modified from reference 220 with permission from Technomic Publishing Co, Inc., copyright 2000. See Table 6 for further details about primer sequences. hel, helicase; pol, polymerase. (B) Schematic representation of the NLV genome from which an internal standard control for genogroup I NLVs was made. The relative locations of selected primers and probes are noted for virus and internal standard (Int. Std.) RNA. The internal standard control RNA yields amplicons that are 123 bp shorter (347 bp) than those from NV genomic RNA (470 bp). A portion of the genomic sequence targeted by virus-specific probes (e.g., SR65 and p116) is not present in the internal standard control, allowing differentiation of virus-specific and internal standard amplicons by nucleic acid hybridization (221).

Very few studies have been reported that describe the quantity of virus genome that must be present in order for a primer pair to detect the virus (159). Instead, most studies only report whether a primer pair can detect a virus strain without regard to the amount of virus present in the sample. However, successful detection of virus may be enhanced when larger quantities of virus are present. For example, Le Guyader et al. (159) noted 10- to 1,000-fold differences in the quantities of several NLV strains that could be detected by two primer pairs, NVp110/p36 and NVp110/p69. In other words, when a large quantity of a virus strain is present, both primer pairs can successfully detect it, but when small quantities of the strain are present, only one primer pair allows successful virus detection. The reason for the differences in virus detection is primer homology, with primers having lower homology requiring the presence of larger quantities of virus for successful amplification to occur. Another approach used to improve the chances of successful amplification has been to lower the primer annealing temperature to as low as 37°C (248). This strategy has the disadvantage of increasing the likelihood that nonspecific amplicons will be generated and may increase the amount of virus that must be present in the sample for successful virus detection.

The use of nested or seminested PCR is another method that has been used to increase the likelihood of detecting NLVs (84, 200, 234). This approach utilizes two rounds of PCR amplification, with one (seminested) or both (nested) primers used in the second round of amplification targeting a region of the genome inside that targeted by the primers used in the initial amplification. This strategy has been reported to be 10 to 1,000 times more sensitive than single-round RT-PCR (84) and has also been used to detect the presence of multiple viral strains within a single sample (200, 234). However, a major disadvantage of this approach is the increased and very real possibility that carryover contamination may occur between samples.

(iii) Other PCR conditions. The conditions (e.g., magnesium concentration and primer annealing temperature) of the RT-PCR assay are dictated, in part, by the primers used. The use of different DNA polymerases during the PCR amplification process has also been reported. Ando et al. (4) used a combination of *Taq* DNA polymerase and *Pwo* DNA polymerase for the amplification of a large (3 kb) fragment of the viral genome. Schwab et al. (222) utilized *Tth* polymerase in place of avian myeloblastosis virus reverse transcriptase and *Taq* polymerase and found that detection of NV in the *Tth* polymerase-

TABLE 6. Common HuCV-specific primers used in RT-PCR assays

Primer	DNA sequence (5′ to 3′)	Genomic location[a]	Sense	Viruses amplified[b]	Reference
p78	GGGCCCCCTGGTATAGGTAA	1682–1701 (2C helicase)	+	NLVs (GI & GII)	248
p80	TGGTGATGACTATAGCATCAGACACAAA	1943–1970 (2C helicase)	−	NLVs (GI & GII)	248
N49(+)	CACCACCATAAACAGGCTG	2215–2233 (2C helicase)	+	NLVs (GI)	181
N49(−)	AGCCTGATAGAGCATTCTTT	2419–2438 (2C helicase)	−	NLVs (GI)	181
p36	ATAAAAGTTGGCATGAACA	4487–4505 (3D polymerase)	+	NLVs (GI & GII), SLVs	248
Sapp36	GTTGCTGTTGGCATTAACA	4487–4505 (3D polymerase)	+	SLVs	180
JV12	ATACCACTATGATGCAGATTA	4552–4572 (3D polymerase)	+	NLVs (GI & GII)	246
P290	GATTACTCCAAGTGGGACTCCAC	4568–4590 (3D polymerase)	+	NLVs (GI & GII), SLVs	125
p3	GCACCATCTGAGATGGATGT	4685–4704 (3D polymerase)	+	NLVs (GI & GII)	188
p69	GGCCTGCCATCTGGATTGCC	4733–4752 (3D polymerase)	+	NLVs (GI & GII), SLVs	248
SR46	TGGAATTCCATCGCCCACTGG	4766–4786 (3D polymerase)	+	NLVs (GI & GII)	3
SR48	GTGAACAGCATAAATCACTGG	4766–4786 (3D polymerase)	+	NLVs (GI & GII)	3
SR50	GTGAACAGTATAAACCACTGG	4766–4786 (3D polymerase)	+	NLVs (GI & GII)	3
SR52	GTGAACAGTATAAACCATTGG	4766–4786 (3D polymerase)	+	NLVs (GI & GII)	3
NI	GAATTCCATCGCCCACTGGCT	4768–4788 (3D polymerase)	+	NLVs (GII)	83
JV13	TCATCATCACCATAGAAAGAG	4858–4878 (3D polymerase)	−	NLVs (GI & GII)	246
E3	ATCTCATCATCACCATA	4865–4881 (3D polymerase)	−	NLVs (GI & GII)	83
NVp110	AC(A/T/G)AT(C/T)TCATCATCACCATA	4865–4884 (3D polymerase)	−	NLVs (GI & GII), SLVs	159
P289	TGACAATGTAATCATCACCATA	4865–4886 (3D polymerase)	−	NLVs (GI & GII), SLVs	125
SR33	TGTCACGATCTCATCATCACC	4868–4888 (3D polymerase)	−	NLVs (GI & GII)	3
p51	GTTGACACAATCTCATCATC	4871–4890 (3D polymerase)		NLVs (GI & GII)	188
p35	CTTGTTGGTTTGAGGCCATAT	4936–4956 (3D polymerase)	−	NLVs (GI & GII), SLVs	248
Sapp35	GCAGTGGGTTTGAGACCAAAG	4936–4956 (3D polymerase)	−	SLVs	20
SRI-2	AAATGATGATGGCGTCTA	5356–5373 (capsid)	+	NLVs (GI)	101
mon381	CCAGAATGTACAATGGTTATGC	5362–5383* (capsid)	+	NLVs (GII)	195
CapIIa	CIAGAATGTAIAA(C/T)GG(G/T)TATGC	5362–5383* (capsid)	+	NLVs (GII)	89
CapIIb	TGIIAGAAAIT(A/G)TTICI(A/G)ACATC(A/T)GG	5559–5584* (capsid)	−	NLVs (GII)	89
CapIa	CICAAATGTAIAATGG(C/T)TGGGT	5647–5668 (capsid)	+	NLVs (GI)	89
SRI-1	CCAACCCA(A/G)CCATT(A/G)TACAT	5652–5671 (capsid)	−	NLVs (GI)	101
mon383	CAAGAGACTGTGAAGACATCATC	5661–5683* (capsid)	−	NLVs (GII)	195
CapIb	TGIIA(A/G)AGIACATTICI(A/T)ACATC(C/T)TC	5844–5869 (capsid)	−	NLVs (GI)	89

[a] Corresponds to the equivalent location within the NV genomic sequence (M87661) (108, 131) except each one marked with an asterisk, corresponds to the equivalent location within the LV genomic sequence (X86557) (55).
[b] GI and GII, genogroup I and II, respectively.

based assay was comparable to that in the two-enzyme system. The use of a single enzyme for reverse transcription and DNA amplification also allowed the use of thermolabile uracil-*N*-glycolase (UNG) for the prevention of carryover contamination (222). dUTP is used in place of dTTP in the RT-PCRs, and the UNG degraded deoxyuracil-containing amplicons added (or carried over) to the reaction tube. The UNG is then inactivated by heating, and the viral RNA is amplified.

(iv) Confirmation of PCR products. A number of methods can be used to interpret the results of a PCR method. One of the simplest is gel electrophoresis. If a band of the size predicted from primer selection is seen following electrophoresis, the PCR is considered positive. This method has been used for NLV RT-PCR assays (10, 84), but it can yield false-positive results (11, 12). Nonspecific amplification of DNA occurs, particularly when more than 30 cycles of amplification are used, and the nonspecifically amplified DNA will occasionally migrate in a fashion similar to that expected for virus-specific amplicons. If this happens, the nonspecific amplification products may be misinterpreted as being virus-specific amplicons. Such nonspecific bands are frequently seen following the assay for NLVs in stool and shellfish samples (12). Because of the potential false-positive results in the visual interpretation of gels, it is essential to confirm the specificity of the amplicons by a second method.

The use of a hybridization assay is probably the simplest approach to interpret and confirm PCR assays. Hybridization assays can be set up in a number of different ways. The most common include dot or slot blot hybridization, liquid hybridization, and Southern blot hybridization. In these assays, a virus-specific probe is labeled and hybridized with the PCR products, and the presence or absence of the label is detected. Some of the more common labels include ^{32}P, digoxigenin, and biotin. One of the limitations of hybridization assays when applied to NLV RT-PCR assays is that the variability of the genomic sequence in the NLVs makes it difficult to select a single or even a small number of probes that can detect all possible NLV sequences (159). Nevertheless, a small number of probes have been effective for the detection of the majority of circulating NLVs when the DNA being amplified was homologous to the polymerase region of the NLV genome (3, 83, 159). The time required to perform the hybridization assay can be shortened without loss of sensitivity by using a direct EIA format. Virus-specific amplicons are captured with a biotinylated probe anchored to a streptavidin-coated, 96-well plate, and the probe-amplicon hybrid is detected using an anti-double-stranded DNA (dsDNA) antibody conjugate (R. L. Atmar and K. J. Schwab, unpublished data). A reverse-line blotting strategy is another approach that has simplified the use of multiple probes at one time and yielded both specificity data and data on the genetic relatedness (genotype) of the HuCV detected to reference strains (247). HuCVs are amplified using biotinylated primers, and virus-specific amplicons are captured during hybridization by one of the multiple probes that are linked to the membrane in individual dots on a blot. Biotinylated products are detected by streptavidin-enzyme conjugates.

DNA sequencing of the PCR products is another approach used to interpret RT-PCR assays (90, 183). Although this method is more laborious and expensive, it also yields the greatest amount of information. A simplified approach in which the biotinylated amplicons are captured on a streptavidin-coated plate and sequenced directly using a virus-specific primer has been described (183). Not only can virus sequence be identified, but the sequence can be compared to those of other samples in epidemiological studies. The ability to distinguish virus strains based on virus sequence is affected by the length of available sequence and the portion of the genome from which the sequence is derived. In general, shorter sequences and sequence derived from the putative polymerase region of the viral genome are less discriminatory than longer sequence and sequence derived from other regions of the viral genome. Thus, some caution must be used when using sequence information to identify potential epidemiological associations. A recent example in which the interpretation of sequence data could be problematic was the description of a worldwide distribution of Lordsdale-like viruses during an 18-month period. Noel et al. (196) noted that virus strains with identical sequences in the RNA polymerase gene (over an 81-base region) were found in multiple countries during 1995 to 1996, and strains with 95.7 to 100% nucleotide similarity over a 277-base region in the capsid gene were seen in different areas of the United States. There were no known epidemiological links between these outbreak strains. Nevertheless, sequence data have been useful in the confirmation of apparent relationships between gastroenteritis cases identified in epidemiological studies and have also demonstrated instances in which more than one NLV was responsible for causing illnesses that had been linked in epidemiological studies (28, 81, 152). Finally, sequence data from two regions of the viral genome have suggested that some caliciviruses may have evolved by undergoing recombination (109, 123, 218, 245). Such events may make it necessary to analyze more than one region of the genome to characterize the relatedness of circulating viral strains in epidemiological studies (218). In addition, a recombination event in an area targeted by a primer or probe could lead to a new strain of virus not detectable using standard primer or probe combinations. Understanding the mechanisms and how frequently recombination occurs will be important in developing strategies for disease prevention or treatment.

(v) Internal standard. As noted above, inhibitors to the RTs and PCRs may persist in samples despite the use of extraction methods designed to remove inhibitors. Failure to detect the presence of persistent inhibitors can cause false-negative results. A number of strategies have been developed for the detection of inhibitors in clinical and environmental samples and include sample dilution, addition of the target nucleic acid to a sample prior to amplification, use of a different primer pair to amplify a second target likely to be present in a sample (e.g., a housekeeping gene), and addition of an internal standard control (221). Sample dilution is effective only when the target nucleic acid is present in sufficient quantity so that it persists in the sample after inhibitors are removed by dilution. This situation has been observed frequently in stool samples containing NLVs; inhibitors are usually removed following a 100-fold dilution of the fecal sample (221). The addition of target nucleic acid to a sample requires that amplification reactions take place in two separate tubes. While this procedure can detect inhibitors, it increases the cost and has the potential to fail to detect problems with amplification that may be unique to a single tube (e.g., failure to add a reagent to an individual tube). Nevertheless, this method has been used to detect the persistence of inhibitors in virus-containing fecal samples after extraction (103). The use of a housekeeping gene as a second target has been most commonly applied to cellular extracts, but this approach has not been used in assays for HuCV detection. The optimal method for detection of competition-inhibition is the use of an internal standard control that is amplified by the same primers used to detect the target viral nucleic acid and that can be distinguished from the target viral nucleic acid (69). Such an internal standard control has been developed for NV (11, 221).

The initial internal standard control developed for NV was a single-stranded RNA that yields amplicons 25 bp shorter than those from NV following amplification with p35 and p36 (11). This internal standard control is effective in detection of inhibitors in shellfish samples being assayed for the presence of NV (11, 12). However, longer times are needed to separate internal standard control- and NV-specific amplicons by agarose gel electrophoresis due to their similarity in size, and the products cannot be distinguished by probe hybridization. A second-generation internal standard control was developed that yielded amplicons 123 bp shorter than those yielded by NV with primers p35 and p36, and this internal standard control can also be used with NVp110 (Fig. 4B) (221). In addition, NV-specific amplicons are detected with use of a probe that targets a portion of the NV genome that was deleted from the internal standard control, and the amplification of the internal standard control is detected with a second probe (e.g., SR48) (Fig. 5). This internal standard has been very effective as an internal positive control when used on stool samples and shellfish samples (221, 223). Its major limitation is its failure to be amplified by primers specific for most genogroup II NLVs. When testing for these viruses, the sample can be divided, with the internal standard control added along with the appropriate genogroup I primers to one portion to determine if inhibitors to amplification are present. An internal standard for both genogroup I and genogroup II viruses is being developed (Atmar, unpublished results).

(vi) Application to clinical and environmental specimens. RT-PCR has become the principal means used by many laboratories for the detection of NLVs and SLVs. The most common clinical sample evaluated by RT-PCR assays is feces. This assay has the advantage of being both sensitive (in terms of detecting small quantities of virus) and broadly reactive (detecting a larger number of strains) compared to antigen detection EIA. Although earlier reports suggested that the antigen detection EIAs for NV had a sensitivity comparable to that of RT-PCR assays (79), later reports from the same laboratory using a more sensitive RT-PCR assay found the latter RT-PCR assay to be more sensitive than antigen detection EIA (221). Similarly, when RT-PCR assays and one or more antigen detection EIAs have been applied to fecal specimens in epidemiological studies, a greater number of positive samples are identified by RT-PCR than by antigen detection EIAs (43, 127, 146, 254).

NLVs have also been detected in vomitus and throat swabs

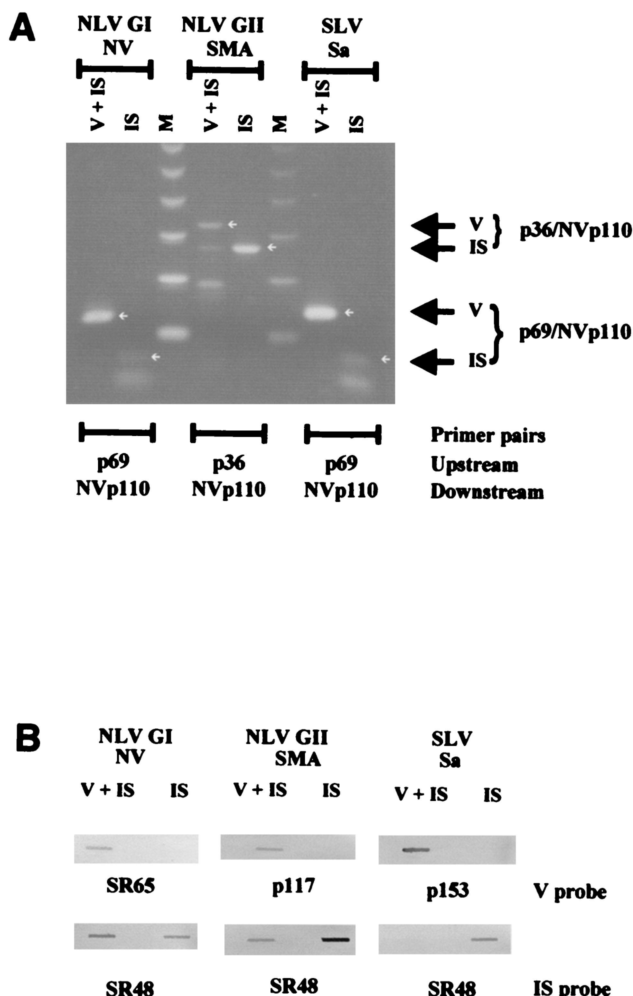

FIG. 5. Representation of RT-PCR detection of NLVs and SLVs by agarose gel electrophoresis (A) and confirmation by slot blot hybridization (B). Virus (V)- and internal standard control (IS)-specific RNAs are visible after amplification of NLVs (NV and SMA) and an SLV (Sapporo virus [Sa]) with primers NVp110 and p36 or p69. Virus-specific amplicons are detected by probes SR65 (NV), p117 (SMA), and p153 (Sapporo virus), while the internal standard control is detected by probe SR48 (3,159).

(85, 184, 200). Barriers to the application of RT-PCR to other samples have been the inhibitory substances and the low concentrations of virus usually present in such samples and have required the development of additional virus concentration or purification steps. Several such assays have been described for different food samples, including deli meats (224), salads (lettuce, melons, and cole slaw) (78), and shellfish (10–12, 78, 84, 120, 157). These assays have been applied successfully for the detection of NLVs in a limited number of studies involving foods (primarily shellfish) (157, 158, 234), water samples (19, 152), sewage (173), and other environmental samples (e.g., swabs collected from areas potentially contaminated by ill persons) (36, 210) associated with outbreaks of viral gastroenteritis.

Use of Newer Diagnostic Tests

The application of the newer diagnostic tests has changed our understanding of the epidemiology of infections caused by HuCVs, especially the NLVs. NLVs are now recognized to be the principal cause of outbreaks of nonbacterial gastroenteritis, and new estimates suggest that they are the most common cause of foodborne illnesses (67, 186, 243). Before the development of the newer assays, NLVs were not thought to be a common cause of gastroenteritis in young children, but several recent studies have found NLVs to be second only to rotaviruses as a cause of viral gastroenteritis in young children, and seroprevalence studies suggest that infection in young children in developed countries is common (25, 82, 163, 205, 206). Although multiple strains may circulate within a community at one time, a single strain or strains within an antigenic cluster may predominate (106, 179, 196). In addition, the duration of virus shedding has been found to be longer than previously recognized (79), providing a potential explanation for the occurrence of foodborne outbreaks traced to postsymptomatic individuals (209, 252).

What approach should be used to determine if an illness or outbreak is associated with HuCV infection? Figure 6 is a schematic of an approach that has been proposed for the collection and testing of specimens in the setting of an outbreak (220). Guidelines for data and sample collection have been published previously (16, 162). Stool and serum samples should be collected from affected individuals as soon as possible after the onset of clinical illness. Although virus is most likely to be detected in stools collected during illness, stool samples collected 1 or 2 weeks after illness onset may still have virus detectable by RT-PCR. VLPs can be used in antibody detection EIAs to determine the presence of virus-specific IgM in individual sera or rising total antibody levels in paired (acute and convalescent phase) sera. The IgM assays are likely to be of greatest value when the initial serum sample is collected more than 10 days after illness onset and when only a single serum sample is available. At present, these tests are available only in a limited number of research laboratories (indicated by the shaded box in Fig. 6), but it is hoped that the reagents will become commercially available. Similarly, the availability of antigen detection EIAs is limited, but fecal specimens can be tested using hyperimmune sera specific for one or several antigenic clusters. If found to be positive, no additional testing is needed. Similarly, some specialized laboratories may be able to evaluate stool specimens using electron microscopy. If the antigen detection EIA or electron microscopy is unavailable or yields negative results, these specimens should be evaluated using RT-PCR assays. RT-PCR is currently the most sensitive diagnostic assay available for the detection of HuCVs, and virus-specific amplicons can be characterized further (e.g., by sequencing) to provide additional information about the virus strain detected. Virus is stable and can be detected by RT-PCR in stool samples stored at 4°C for several months (at least) or at −70°C for more than 10 years. The ability of RT-PCR assays to detect virus collected or stored under suboptimal conditions (e.g., in bacterial transport medium) is not known but could be evaluated in future studies. Potential vehicles of transmission (food and water) may be collected and analyzed by RT-PCR and sequencing of virus-specific amplicons to establish routes of transmission. RT-PCR assays are being performed by an increasing number of diagnostic laboratories. Thus, the equipment needed to perform such assays is more readily available,

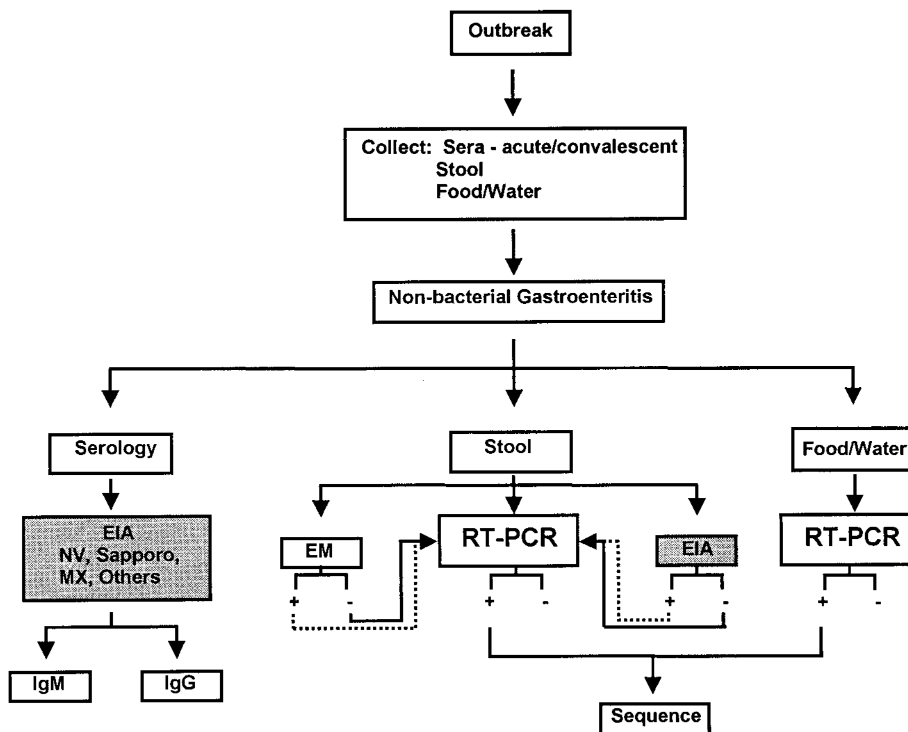

FIG. 6. Schematic of evaluation strategies for the identification of infection caused by HuCVs. Once an outbreak is identified, clinical samples (serum or stool) and potential vehicles of transmission (e.g., water and food) are collected. Stool samples can be evaluated by any of three methods: electron microscopy (EM), RT-PCR, or EIA. If EIA or EM is positive, calicivirus infection is confirmed. Samples negative by EIA or EM and those for which further characterization is desired can be evaluated by RT-PCR. Virus-specific amplicons from stool or food and water samples may be sequenced and compared to determine the similarity of the strains. Serologic studies may also be performed using a number of different VLP antigens. IgM tests are performed on single sera, and IgG tests are performed on paired sera. The shaded boxes show those tests that are currently only available in selected research laboratories. Reprinted from reference 220, with permission from Technomic Publishing Co, Inc., copyright 2000.

and the reagents needed for these assays can be purchased commercially.

Future Directions

The identification of a common epitope shared by genogroup I NLVs belonging to several different genetic clusters suggests that the development of a broadly reactive antigen detection EIA will be possible. Future studies will determine if such epitopes exist. As a greater number of VLPs representing distinct genetic clusters within the viral genera are developed, further refinements of both antigen detection and antibody detection EIAs can be expected. In turn, these assays may be used to further define the circulation of specific strains over time and increase our understanding of the epidemiology and transmission of HuCVs (106). RT-PCR assays, currently the most widely used diagnostic assays for HuCVs, will be refined through the further development of procedures that simplify viral nucleic acid extraction, the RT-PCR process, and the detection of virus-specific amplicons. Such refinements will allow the testing of a greater range of samples, including food and environmental samples implicated in outbreaks of gastroenteritis, and the development of strategies to decrease the spread of these viruses within the community. Additional sequence data may clarify the occurrence and frequency of recombination events among circulating viruses and increase our understanding of the replication and evolution of these viruses (109). Finally, efforts to cultivate HuCVs in vitro will continue. The development of a culture system for these viruses will lead to significant increases in our understanding of virus replication, immune correlates of protection from infection, and the environmental factors that lead to virus inactivation (loss of infectivity).

CONCLUSIONS

The cloning and sequencing of NV and subsequently other HuCVs has led to the development of several new diagnostic assays. Antigen detection EIAs using polyclonal animal sera produced by hyperimmunization with HuCV VLPs are more sensitive than previously available RIAs, but their utility has been limited due to their high specificity, as each assay only detects viral strains closely related genetically to the strain used for hyperimmunization. The ability to produce MAbs easily by immunization and screening with VLPs has led to the identification of a common epitope shared by genogroup I NLVs, suggesting that it will be possible to develop a more broadly reactive antigen detection EIA. HuCV VLPs also have been used to develop antibody detection EIAs that are more sensitive than previously available RIAs and EIAs based on the use of human reagents. Both class and subclass immune responses can be detected, and these responses occur as early as 8 days following infection. The antibody detection EIAs are more

broadly reactive than the antigen detection EIAs, more readily detecting infections caused by heterotypic NLVs. RT-PCR assays are the most sensitive of the newly developed assays, detecting both smaller quantities and a broader array of HuCVs than either of the other two groups of new assays. Although no single primer pair can detect all HuCVs and the performance of RT-PCR assays for HuCVs is not yet standardized, several approaches have been successfully applied in both epidemiological studies and outbreak investigations. Another advantage of RT-PCR assays is their applicability to environmental specimens. These new assays have significantly increased the recognition of HuCVs as causes of sporadic and outbreak-associated gastroenteritis, and new information about factors leading to disease transmission may permit development of new public health measures to decrease this common disease.

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