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Nonculturable Agents of Viral Gastroenteritis

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Gastroenteritis is one of the most common diseases of humans, resulting in illness that ranges from mild diarrhea to profound dehydration and death (1). Viruses have been suggested as a cause of gastroenteritis since Gordon et al. (2) induced illness in volunteers given stool filtrates 50 years ago. Twenty-five years later, studies by Kapikian et al. (3) provided the first clear demonstration of the causal relationship between a virus (Norwalk virus [NV]) and gastroenteritis by using immune electron microscopy (IEM) to detect the presence of viral particles in the stools of individuals from an epidemic outbreak of gastroenteritis. Virus was detected in the stools of affected subjects, but not in non-ill controls, and serologic immune responses to the viral agent also were demonstrated in affected subjects. Since that time, a number of other viruses have been shown to cause gastroenteritis in humans (Table 1). Criteria to prove a virus causes gastroenteritis include identification of the virus in subjects with diarrhea more frequently than in controls, demonstration of an immune response to the specific agent, and demonstration that the onset and termination of illness corresponds with the period of virus shedding (4). Several viruses that cause diarrhea in animals have been found in the stools of children or adults with diarrhea, but these viruses have not yet fulfilled the criteria necessary to assign them an etiologic role in gastroenteritis in humans (Table 1).

Study of the rotaviruses, enteric adenoviruses, and astroviruses, has been greatly facilitated by the use of cell culture to make viral proteins and genetic material available for use in diagnostic assays. Other gastroenteritis or probable gastroenteritis viruses remain noncultivable (Table 1). The study of human caliciviruses has been difficult due to the inability to grow these viruses in cell culture. However, the first successful cloning of the NV genome in 1990 (5) opened up new approaches to study these noncultivable human pathogens and led to proof that this antigenically diverse group of viruses, previously called small, round-structured viruses and human caliciviruses are members of the same virus family. Cloning, sequence analysis, and expression of the NV capsid protein has impacted both clinical and basic knowledge about these viruses (Figure 1). This article describes the structure and genome organization of the human caliciviruses that cause gastroenteritis, the clinical and epidemiologic features of these viruses, and new methods for the laboratory diagnosis of infection with these viruses.

Virus Structure and Genomic Organization

Human caliciviruses (HuCVs) are 26 to 35 nm nonenveloped, icosahedral viruses made from a single capsid protein, and they have a single-stranded, positive sense RNA genome that ranges in size from approximately 7.3 kb to 7.7 kb in length. The name calicivirus is derived from the Latin *calyx*, meaning "cup" or "goblet," and refers to the cup-shaped depressions visible by electron microscopy on the surface of morphologically-typical caliciviruses [Figure 2; (6)]. These depressions are frequently not clearly visible (e.g., for NV), although structural studies have demonstrated their presence (7). Because of these structural differences and the previous use of IEM to detect these viruses, many of the HuCVs were initially referred to as small, round-structured viruses (SRSVs). NV, one of the viruses previously called a SRSV, has been designated the prototype human calicivirus based on its genome organization and structure being similar to those of animal caliciviruses (8). Characterization of more than 50 SRSVs has shown them all to be HuCVs. In order to simplify communication about different

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small, round fecal viruses, it has been proposed that these viruses be referred to in the following fashion: type of virus/name of virus/strain designation/year of isolation/country of isolation. Thus, NV is designated HuCV/NV/8FIIa/68/US.

The inability to cultivate the HuCVs and establish neutralization assays has prevented the definition of specific serotypes; however, at least five different serotypes are thought to exist based on early human cross-challenge studies and comparisons of the IEM and enzyme-linked immunosorbent assay (ELISA) reactivities of several prototype virus strains. The different serotypes are represented by NV, the Hawaii agent (HV), Snow Mountain agent (SMA), the Taunton agent, and the Sapporo virus. The reactivity of HuCVs with convalescent human sera obtained from different outbreaks of gastroenteritis also has been used to classify viruses by solid phase immune electron microscopy (SPIEM), and up to six different serogroups have been suggested (9). This method is limited by the availability of specific human sera for use in the assays.

Three distinct genogroups of HuCV have been identified based on the analysis of the partial or complete nucleotide and predicted amino acid sequences of at least 50 different HuCVs (10-16). NV is a genogroup I virus, HV and SMA are genogroup II viruses, and the Sapporo virus is a genogroup III virus. Genogroup I and II viruses have three open reading frames (ORFs): the first ORF encodes a polyprotein with regions of amino acid similarity to the picornavirus 2C helicase, 3C protease, and 3D polymerase; the second ORF encodes the capsid protein; and the third ORF encodes a highly basic protein of unknown function. In genogroup III viruses, there are only two ORFs; the first ORF is longer than in genogroup I and II viruses and contains the sequence encoding the nonstructural proteins and the capsid protein. Characterization of clinical HuCV

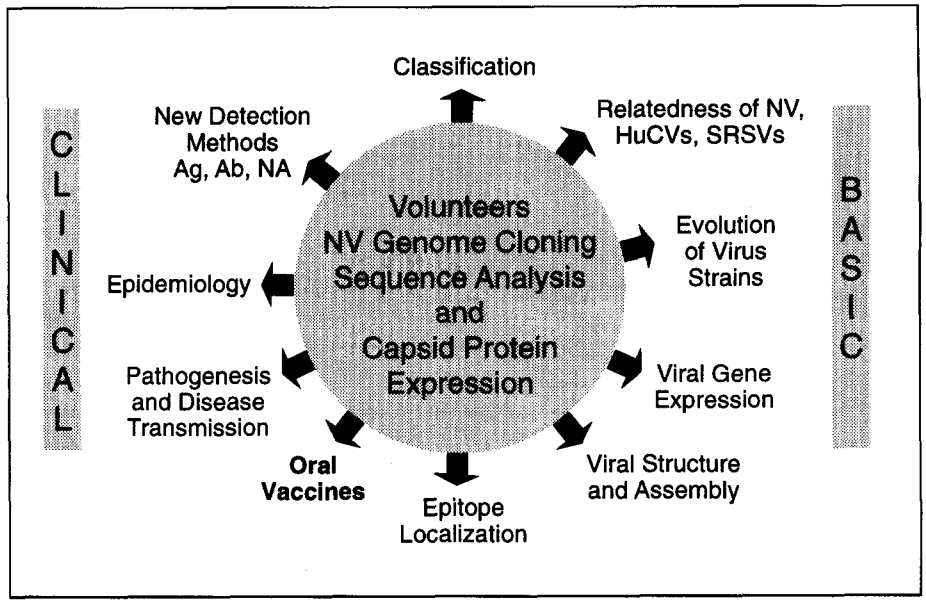

Figure 1. Impact of cloning on knowledge about Norwalk Virus and Human Caliciviruses. Cloning and sequencing of the Norwalk virus genome has resulted in studies that have changed our understanding of a variety of clinical and basic topics about these viruses.

Table 1. Enteric viruses and causal relationship to diarrhea

	Cultivation Reported[b]
Causal relationship demonstrated[a]	
Rotaviruses	yes
Human caliciviruses (e.g., NV, SMA, Hawaii agent, Sapporo HuCV)	no
Astroviruses	yes
Enteric (group F) adenoviruses	yes
Candidate agents — etiologic relationship not yet determined	
Coronaviruses	yes
Echovirus type 22	yes
Picobirnaviruses	no
Pestiviruses	no
Toroviruses	no
Other agents — causal relationship unclear[c]	
Non-group F adenoviruses	yes
Coxsackie A and B viruses	yes
Echoviruses	yes

[a]Listed by relative clinical significance
[b]Cultivation of a virus from human enteric sample
[c]Present in stools of non-ill individuals

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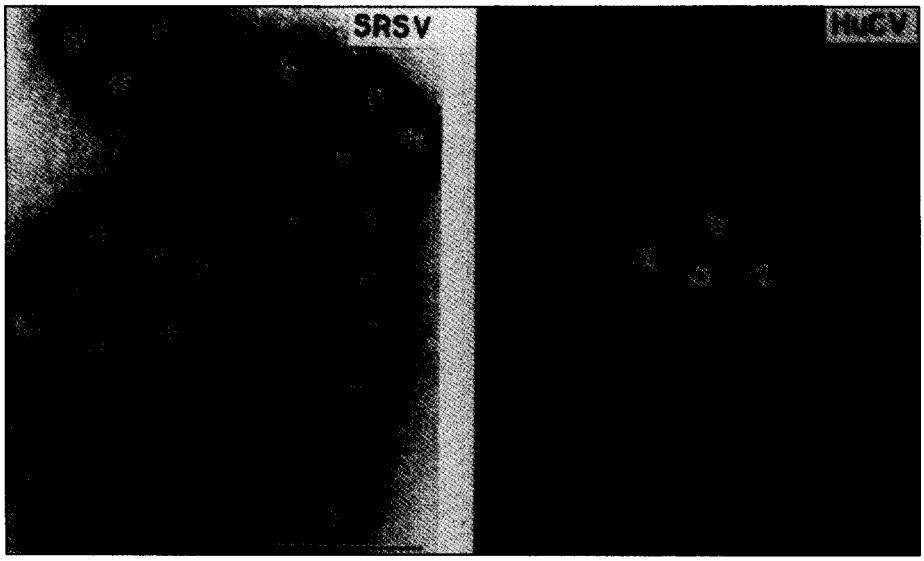

Figure 2. Electron micrographs of different human caliciviruses in stools. A. Norwalk virus, previously called a small, round-structured virus. B. Human calicivirus Sapporo, a virus with typical calicivirus structure.

strains into different genogroups based on sequence analysis of the RNA polymerase region has not always agreed with the results obtained by SPIEM; for example, different strains with the same SPIEM type have been found to belong to different genogroups (15). The reason(s) for these differences is not yet known.

Epidemiology

HuCVs are the major cause of epidemic gastroenteritis in both developed and developing countries. Outbreaks have been described in families, day care centers, schools, the military, recreational camps, cruise ships, communities, hospitals, and nursing homes. The vehicle of transmission may be either food or water and has included uncooked or undercooked shellfish, salads (e.g., fruit, potato, tossed), cold foods (e.g., celery, sandwiches, ham), bakery products (frosting), drinking water, ice, and swimming water (6). Transmission by the airborne route also has been suggested (17,18).

The morphologically-typical HuCVs were first detected in infants and young children (19), and seroprevalence surveys have shown HuCV infection may occur at an early age, particularly in developing countries (6). However, infection has most commonly been recognized in school-aged children and adults. Illness tends to be mild and self-limited, in contrast with infantile gastroenteritis caused by rotavirus, which is often severe and may be life-threatening. However, even epidemic illness of short duration as is seen with HuCVs can cause significant morbidity in some populations, such as the military (20). Nosocomial transmission in the health-care setting may occur (21). A greater appreciation of the significance and impact of HuCV infections as a cause of disease should result from the wider application of the newer diagnostic assays described below. Use of these assays is changing our understanding of the epidemiology and natural history of these infections. For example, new information has documented infections and disease in younger children and immunocompromised children. In addition, new studies have found that virus is shed for longer periods of time than previously appreciated and from asymptomatic individuals. This latter point is important as it may explain virus transmission in the absence of an identifiable source.

Clinical Illness

HuCV infection is characterized by the acute onset of vomiting or diarrhea, or both. There is considerable variability in the pattern of clinical symptoms between patients. The illness in volunteers challenged with NV ranges from a vomiting illness with no diarrhea to a diarrheal illness with no vomiting. Nausea, abdominal cramps, and headache also are common symptoms. Low grade fever is seen in a minority of patients. Infection is asymptomatic in as many as a third of subjects based on fecal virus excretion and serologic studies. There is no prodrome, the mean incubation period is 24 to 48 h, and symptoms last from 12 to 60 h.

HuCV infections should be suspected in the investigation of gastroenteritis outbreaks if the following conditions are met: (i) no bacterial or parasitic pathogen is identified; (ii) vomiting is present in more than 50% of cases; (iii) the mean or median illness duration is 12 to 60 h, and (iv) the incubation period is 24 to 48 h. These criteria have been useful in several epidemiologic investigations (22). A definitive diagnosis requires virus identification, as outlined below.

Diagnostic Assays

Until recently, diagnostic assays relied on the use of human reagents (sera) for virus detection, limiting the sensitivity and availability of these assays. The cloning of NV and other HuCVs have made available newer reagents and in greater quantities. The new reagents have been used to develop three types of new assays: antigen detection ELISAs to detect virus or viral antigen in stool extracts, antibody ELISAs to detect seroresponses, and reverse transcription-PCR (RT-PCR) to detect viral RNA. All are described below.

Antigen Detection

Electron Microscopy (EM) of unconcentrated stool samples is of limited value due to the very low concentration of virus usually present in stool samples and the presence of other small, round, non-viral objects in stool. A vast amount (>50%) of NV antigen in stool also is soluble antigen that is not detected by EM or IEM. IEM was the first immunologic method used to detect NV infection, and it increases the sensitivity and specificity of detection by electron microscopy. However, it may be negative in more than half of stool samples collected within the first 72 h of illness (24). IEM is expensive, requires a highly skilled microscopist, and also requires the availability of suitable reference antisera, further limiting its usefulness.

Radioimmunoassays (RIAs), and

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subsequently ELISAs, were initially developed in the 1970s using acute and convalescent sera from infected individuals as capture and detector antibodies (25-27). These assays were at least as sensitive as IEM and easier to perform, but their use was limited to research laboratories due to the lack of availability of reagents. Recently, the human reagent-based assays have been replaced by assays that use hyperimmune polyclonal or monoclonal sera produced in different animal species to viral-specific proteins expressed from cloned genes (28). For example, expression of the capsid protein of NV in a baculovirus expression system results in the formation of virus-like particles (VLPs) that are morphologically similar to native virus (23). These VLPs have been used to produce hyperimmune sera in different animal species and monoclonal antibodies, and these sera have been used to develop new diagnostic assays. An assay using hyperimmune sera produced against NV VLPs is more sensitive than RIA and has almost equivalent sensitivity to early RT-PCR assays used to detect the virus genome in stool specimens from NV-challenged volunteers, probably due to the presence of large amounts of soluble capsid protein in the stool (28). However, unlike the assays using human reagents, the antigen-detection assay using hyperimmune animal antisera is quite type-specific, detecting only those HuCVs most closely related to the type of VLP used to make the anti-serum (14). Similar findings have been obtained with hyperimmune sera made to HuCV/NV/8FIIa/68/US and to HuCV/Mexico virus/MX/89/Mexico (MX), a genogroup II virus, and several other HuCVs (14,29-31). These findings most likely are due to the induction of cross-reactive antibodies in convalescent sera, reflecting an individual's past infections with HuCVs, while the immunized animals have had no past experience with HuCVs. The high specificity of the new assays limits their usefulness for detection of all HuCV infections. Polyclonal and monoclonal antisera that are reactive with at least three serotypes of HuCVs have been produced, and assays using these sera show promise of being broadly cross-reactive (32,33). These findings suggest that a broadly reactive antigen detection assay will be developed for use in the near future.

Antibody Detection

Many of the early antigen detection assays were modified for the detection of antibody. Thus, IEM was used to evaluate the relative ability of acute and convalescent sera to immunoprecipitate virus, and RIAs and ELISAs were used in blocking assays (3,25-27). With the production of VLPs in baculovirus expression systems, antibody ELISAs have been developed using VLPs as antigen to coat the wells of ELISA plates. A four-fold or greater increase in serum antibody titer between acute and convalescent sera has been used to define recent infection. These assays are more sensitive than IEM or RIA blocking assays (34). The antibody ELISA using VLPs as antigen is able to detect infection with different HuCV serotypes, although it is most sensitive for the detection of infection with virus most closely related to the VLP type (35). The utility of these new antibody assays has been demonstrated in both small and large scale epidemiologic investigations. ELISA antibody assays to detect IgM, IgA, and IgG subclasses also have been described (35-37).

RT-PCR

At present, RT-PCR is the most sensitive method for the detection of infection with HuCVs. Most RT-PCR assays use primer pairs that amplify the RNA-dependent, RNA polymerase region of the genome (11-15,38-41), although other areas of the genome also have been targeted (12,14,42). The genetic diversity of the HuCVs has prevented the development of a universal primer pair to detect all HuCVs. However, viruses in all three genogroups can be detected using a single degenerate primer for cDNA synthesis and two additional upstream primers during PCR (41). The genetic diversity of HuCVs also has made the confirmation of the specificity of the RT-PCR assay difficult, with as many as ten oligoprobes not being able to identify all HuCV PCR products. Sequencing of the PCR products is an alternate confirmatory approach that is now feasible by automated sequencing of PCR products (43).

Stool contains substances that inhibit the enzymes used in the RT-PCR reaction, and a number of extraction methods have been used to remove these inhibitors (38,39,44). We have recently found that the combination of dilution of the stool sample with heat release of the viral RNA is as effective for the detection of virus as more conventional extraction methods (45). The use of an internal standard RNA control is important to allow the detection of inhibitors in a sample and false-negative results (46). Methods for the RT-PCR detection of virus in shellfish have been developed and used to detect HuCVs in shellfish associated with a gastroenteritis outbreak (46,47).

RT-PCR has a theoretical ability to detect fewer than 10 copies of viral genome, and in practice has detected less than 50 copies of NV genome (46). This is approximately 200-fold more sensitive than the level of detection expected for antigen detection ELISAs (~10^4 virions/ml) and 20,000-fold more sensitive than IEM (>10^5 virions/ml) (6). The exquisite sensitivity of the RT-PCR assay requires that extreme caution be maintained to prevent carryover contamination.

Conclusion

The development of new assays has expanded our ability to detect infection due to HuCVs and extended our knowledge about the epidemiology of these infections. New information about the epidemiology of HuCV infections has shown that infections with HuCVs are more widespread than previously recognized, and children are more frequently infected. Further improvements in the assays described above should increase the general utility of the assays, and lead to their greater availability in the future. Further use of these assays and access to more precise knowledge of the epidemiology and natural history of these infections should allow better prevention/intervention strategies to be developed.

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Editorial

The Non-Physician Expert in Medical Malpractice Litigation

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Medical malpractice litigation is an interesting and challenging field for the biomedical scientist. It offers the opportunity to apply his or her specialized knowledge and experience in the legal arena by participating in the defense of a physician unjustly accused of injuring a patient or in supporting the claim of a patient who has been harmed by the negligence of a physician.

Medical malpractice litigation revolves around the theory of negligence. To prove negligence the plaintiff must establish: (i) a duty owed to the patient by the treating physician; (ii) a breach of that duty; (iii) injury or damage; and (iv) proximate cause. Breach of the duty owed to the patient requires proof of the acceptable standard care, a standard that has been defined as "what the reasonable person of ordinary prudence would do under like circumstances."

With the possible exception of limited circumstances that are self-evident, known as *res ipsa loquitor,* an expert witness is required to define for the court the appropriate standards of care, and possibly testify as to how the defendent physician's actions conformed or deviated from these standards.

Federal Rule of Evidence (F.R.E). 702 states: If scientific, technical, or other specialized knowledge will assist the trier of fact to understand the evidence or to determine a fact in issue, a witness qualified as an expert by knowledge, skill, experience, training, or education may testify in the form of an opinion or otherwise.

Expert witnesses asked to testify in medical malpractice cases are usually physicians who specialize in a medical discipline related to the case. However, biomedical scientists who are not physicians can often provide cogent and relevant information and/or testimony. Qualifications to be accepted as an expert witness usually include a doctoral degree in a basic medical science, teaching experience in a medical facility, publications in scientific journals, and membership in professional societies. Clinical chemists, pharmacologists, physiologists, microbiologists, immunologists, and others may furnish valuable input by acting as a *testifying expert* or as a *litigation consultant.*

The testifying expert plays an active role in the legal controversy. He/she will be examined by the opposition at deposition and possibly later at trial. All of the expert's activities relating to the case, as well as his or her work products, are discoverable. The expert's effectiveness as a witness during trial will be as much determined by his or her demeanor and presentation in court as by the factual material presented.

Since 1993, many courts have adopted the *Daubert* standard which holds that scientific testimony must be screened by the trial judge to assure its *relevance* and *reliability*. The Federal Judicial Center's *Reference Manual on Scientific Evidence*, intended as a guide for judges, lawyers, and experts, asks a fundamental question: Is the expert qualified?

The opposition will try to negate or at least lessen the impact of the expert's testimony by raising the question of his/her qualifications. A serious attempt will be made to disqualify the non-physician expert on the basis of a lack of clinical experience, training, or education.

The litigation consultant who is not called as an expert witness is immune from these challenges. Like a coach at an athletic event, he/she does not participate in the contest but remains on the sidelines advising and counseling. The consultant is not subject to examination by the opposition, and his or her activities and work products such as reports to the attorney, are not subject to discovery.

The following three cases illustrate how a non-physician expert effectively functioned as a consultant in malpractice actions:

Case 1. The plaintiff cut his leg on a fence while chasing his horse. The emergency room physician treated the wound and sent him home. He returned several hours later in great pain, and was again treated by the physician. The following day the plaintiff consulted a surgeon who admitted him to the hospital and performed extensive surgery on the man's leg. The surgeon remarked that the emergency room physician had not properly cleaned and drained the wound. The patient sued the emergency room physician for malpractice. Acting as a consultant for the defense, the non-physician expert, familiar with minor surgical procedures, reviewed the emergency room physician's and nurse's notes, and was able to advise the defense attorney that the treatment provided by the physician conformed to or exceeded the standard of care in such cases. The expert relied upon several textbooks of surgery.

Case 2. A women fell and injured her knee. Following treatment by a physi-

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