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CALICIVIRUS INFECTION AMONG

HOSPITALIZED CHILDREN

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per optar al grau de Doctor en Medicina.

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GLOSSARY OF ABBREVIATIONS

bp:	Base pair
CDC:	Center for Disease Control
CV:	Calicivirus
ddNTP:	Dideeoxyribonucleotide triphosphates
dNTP:	Deoxyribonucleotide triphosphates
DSV:	Desert Shield virus
EIA:	Enzyme immunoassay
ELISA:	Enzym linked immuno assay
EM:	Electron microscopy
HuCV:	Human calicivirus
ICTV:	International Committee on Taxonomy of Viruses
IEM:	Immune electron microscopy
IL-1:	Interleucine 1
NLV:	Norwalk-like virus
NV:	Norwalk virus
ORF:	Open reading frame
PAF:	Platelet activating factor
PCR:	Polymerase-chain reaction
RIA:	Radioimmune assay
RT:	Reverse transcription
RT-PCR:	Reverse transcriptase-polymerase chain reaction
SLV:	Sapporo-like virus
SMV:	Snow Mountain virus
SRSV:	Small round structured virus
TNF:	Tumor necrosis factor
VES:	Vesicular exantema of swine virus
VLP:	Viral like particle
WHO:	World Health Organization

CONTENTS

1. <u>INTRODUCTION</u>

1.1. ACUTE GASTROENTERITIS

Acute gastroenteritis is an age old syndrome physicians encounter daily. Diarrheal diseases have been recorded since the beginning of civilization as shown in the hieroglyphs for diarrhea and watery diarrhea in the Ebers papyrus and the Hearst papyrus (3300 BC) [1].

1.1.1. DEFINITION

Acute gastroenteritis describes a clinical syndrome consisting of a constellation of signs and symptoms, usually of less than two weeks duration. Affected individuals typically have combinations of one or more of the following signs and symptoms: diarrhea, vomiting, fever, abdominal cramps, dehydration, irritability, malaise, and anorexia. The manifestations vary among individual patients and also depend on the duration and severity of symptoms. Some patients develop metabolic acidosis caused by bicarbonate loss and dehydration. These symptoms can result from gastrointestinal infection by viral, bacterial, fungal, or parasitic organisms although they can also be due to noninfectious causes such radiation, toxics, hypersensitivity, immune reactions,…

1.1.2. ETIOLOGY

During the last three decades, a dramatic increase has occurred in the number of newly recognized etiologic agents of acute gastroenteritis. Before 1970, a pathogen could be identified in fewer than 10% of patients hospitalized with diarrhea in the United States. The remaining 90% of cases without an identified pathogen constituted a "diagnostic void" consisting of various idiopathic, ill-defined conditions, such as the diarrhea of weaning, malnutrition, or old age. Since 1970, more than 20 different microorganisms, including bacteria, parasites, and viruses, have been identified as etiologic agents (Table 1) and most cases of acute gastroenteritis are now presumed to have infectious etiology. Nonetheless, because only a few assays for etiologic agents are applied, the responsible pathogen is still identified in only a minority of cases and those that are identified vary from site to site and study to study. Furthermore, the reported prevalence of an enteric pathogen more often reflects the convenience and availability of diagnostic tests

specific for particular organisms than the true prevalence of the agent. Consequently, those pathogens most difficult to detect are considered uncommon and relegated to the diagnostic void. For example, a review of the etiology of nearly 7,500 foodborne outbreaks of gastroenteritis reported to the CDC from 1973 to 1987 identified 25% as bacterial; 1.8% as viral (Norwalk-like viruses and hepatitis A virus), and 1.8% as parasitic. The remainder 62% were of unknown cause[2]. An investigation including over 30,000 patients hospitalized for diarrhea between 1990 and 1992 at 10 sentinel centers in the USA found that 5.9% had identified bacterial pathogen, leaving 94% in the diagnostic void that in this study included viral and parasitic pathogens[3].

Table 1: Etiologic Agents of Acute Gastroenteritis

Bacteria	**Protozoa**
Aeromonas species	*Cryptosporidium* species
Bacillus cereus	*Cyclospora* species
Campylobacter jejuni	*Entoameba histo lytca*
Clostridium difficile	*Giardia lamblia*
Clostridium perfringens	*Isospora belli*
Escherichia coli	*Microsporidia*
Plesiomonas shigelloides	
Salmonella species	**Helminth**
Shigella species	*Capillaria philippinensis*
Vibrio cholera	*Schistosoma* species
Vibrio parahaemolyticus	*Strongyloides stercoralis*
Yersinia enterocolitica	*Trichinella spiralis*
	Trichuris trichiura
Virus	
Enteric adenovirus (types 40 and 41)	
Astrovirus	
Calicivirus	
Rotavirus	

1.1.3. EPIDEMIOLOGY AND IMPACT

Diarrheal disease is a leading cause of mortality and morbidity among children worldwide. In developing countries, diarrheal disease constitutes a very important public health problem with over 1 billion illness episodes and over 4 million deaths annually in children less than 5 years of age [4]. In developed countries, mortality from acute gastroenteritis is much less frequent. Diarrheal disease, however, despite major public health advances to improve the quality of food, water, and sanitation, remains second only to the common cold in frequency of occurrence, accounting for significant morbidity and expense [5]. The 250-350 million episodes that occur each year in developed countries result in approximately 450,000 hospitalizations in adults, 160,000 hospitalizations in children and over 400 deaths[6-8]. In terms of health care delivery, 10-20% of hospitalizations among children younger than 5 years of age [9] and 1.5% of those among adults are associated with gastroenteritis [6,10]. One in 25 American children will be hospitalized for diarrhea by the age of 5 years, and approximately 1 in 8 American adults admitted to a hospital will be discharged with a diagnosis of primary or secondary AGE during their adult years [6]. Surveys in the United States suggest that nearly every American will have an episode of gastroenteritis each year [11], and the rate is higher for children less than 5 years of age and for the elderly, the latter having the greatest risk of a fatal outcome [10].

In addition to acute morbidity and mortality, some cases of infectious diarrhea result in serious long-term sequelae, such as hemolytic uremic syndrome with renal failure following enterohemorragic *E. coli* infections, Guillain-Barre syndrome following *C. jejuni* infection, and malnutrition with or without diarrhea following infection with enteroaggregative *E. coli*, *Cryptosporidium* species, or perhaps other enteric infections.

The interaction between gastroenteritis and malnutrition, particularly around the period of weaning, is a crucial factor affecting morbidity and mortality in the non-industrialized world. Gastrointestinal infections are more common in malnourished children: changes in the mucosa, the lymphoid tissue, and in the permeability are likely to prolong periods of infectivity by a variety of pathogens and could also increase the risk of systemic spread. The potentially huge impact, in

the developing world, of long-term disability caused by repeated early childhood enteric infections is also an important fact to considerer when assessing preventive strategies.

1.1.4. PATHOPHYSIOLOGY AND CLINICAL MANIFESTATIONS

The term "gastroenteritis" is derived from the Latin word *gastro* for stomach, *enteric* for intestinal and the termination *itis* for inflammation.

Different events, such as infections, ischemia, radiation, and chemical toxins, induce an inflammatory reaction that is clinically, endoscopically, and histologically similar. The seemingly identical inflammatory response is explained partly by the similarity between the proinflammatory cytokines, the patterns of leukocyte migration, and the inflammatory mediators induced by these initiating events. Gastrointestinal inflammation produces an organ-level physiologic response consisting primarily of increased motility and secretion in an attempt to wash out the offending event. Increased secretion and motility result in cramps and diarrhea which are prominent clinical features of enteric infections[12]. There is also a systemic response to gastrointestinal inflammation with the release of cytokines and other inflammatory mediators that will cause constitutional symptoms like fever, malaise, and anorexia.

1.1.4.1. Diarrhea

Definition of diarrhea

Diarrhea was defined by Roux and Ryle, at the turn of the last century, as 'the too rapid evacuation of too fluid stool' [13]. The ambiguity of this definition reflects the difficulty of characterizing it. Normal stooling frequency ranges from three times a day to three times a week; the wide interval of normality and the interpersonal variation makes it difficult to exclusively use stooling frequency as a definition of diarrhea. On the other hand, individuals rarely report increases in frequency alone as diarrhea; a decrease in stool consistency or increased fluidity, as well as stools that cause urgency or abdominal discomfort, are more likely to be termed diarrhea by patients. Although stool consistency probably best defines diarrhea, it cannot be easily measured.

The physician or clinical investigator often chooses to define diarrhea as a physical sign (24-hour excretion weight or volume higher than 200 g for adults or 10g/kg in infants) rather than a symptom; by using this definition, 20% of patients with loose stools will be misdiagnosed.

Pathophysiology of diarrhea

In the early part of the 20th century, diarrhea was thought to be caused primarily by abnormal gastrointestinal muscle contraction. Although the role of gastrointestinal motility in diarrheas is still not completely understood, most of the diarrheal conditions have shown alterations of both intestinal fluid and electrolyte transport and smooth muscle functions, often with the induction of propagative forms of intestinal motility (Figure 1).

There are two general categories of diarrheal pathophysiology: malabsorption and secretion, also referred to as osmotic and secretory diarrheas, respectively.

a) Pathophysiology of osmotic diarrheas

When a non-absorbable solute reaches the jejunum it results in fluid entry into the small bowel rendering the intralumenal solutions isosmotic with the plasma. Intralumenal Na^+ concentration drops below 80 mM and the permeable jejunum cannot absorb Na^+ against such a steep lumen-to-plasma gradient. If the non-absorbable solute is also non-digestable (i.e. magnesium), some Na^+ and water

Figure 1: The diarrhea spiral (Yamada 1992)
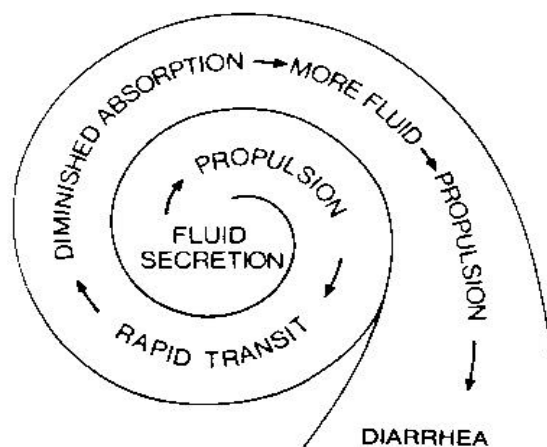
may be absorbed by the colon, which can concentrate Na^+ from a lumenal concentration below 30 mM but it cannot absorb all the excess of water. If the unabsorbed solute is a carbohydrate that can be digested by colonic bacteria, it is catabolized to short-chain fatty acids (organic anions), which obligates retention of inorganic cations, significantly increasing the number of osmotically active particles in the colon and therefore the solute load, promoting the movement of more fluid into the colon. Although some of the organic anions and fluid are absorbed as they traverse the colon, the unabsorbed carbohydrate, the organic anions with their obligate cations, and fluid are excreted in the stool.

The osmotic solutes causing osmotic diarrheas can be derived from either exogenous (ingested) or endogenous sources and may result from congenital or acquired malabsorptive disease.

b) Pathophysiology of secretory diarrheas

In the years following 1965, scientists discovered the ability of the intestine to secrete as well to absorb fluid and electrolytes. After this discovery, active secretion (due to neurotransmitters, hormones, bacterial endotoxines and cathartics) was added to the failure to absorb the major electrolytes in the lumen (Na^+, K^+, Cl^-) due to epithelium damage as physiopathologic mechanism of diarrhea. The stimuli causing secretory diarrhea may be of exogenous origin (laxatives, medications, toxins) or endogenous (hormone-producing tumors, dihydroxy bile acids).

Although initially it was thought that bacterial enterotoxins caused secretion only by a direct effect on the enterocyte receptors, 50% of the intestinal secretion initiated by bacterial enterotoxins in vivo comes about from stimulation of receptors on enterochromaffin cells which release hormones that activate the enteric nervous system, secondarily stimulating the enterocyte[14].

c) Pathophysiology of inflammatory diarrheas

Some diarrheas are characterized by enterocyte damage and death, in which both malabsorption and secretion occur. This is the case of some infectious diarrheas.

When colonization, adherence, or epithelial invasion by microorganisms occurs, there is a release of various cytokines and inflammatory mediators from the

epithelium and from the lumenal microorganisms inducing activation of phagocites. Release of mediators such as prostaglandins, leukotrienes, platelet activating factor (PAF), and hydrogen peroxide from phagocytes induces intestinal secretion by acting on the enterocyte and also by activating enteric nerves (Figure 2). If the chemotactic substance released by the epithelium or by lumenal microorganisms is also sufficiently concentrated in the lumen of the bowel, neutrophils may cross the epithelium to form a crypt abscess.

Figure 2: Pathophysiology of inflammatory diarrhea (Yamada, 1999)
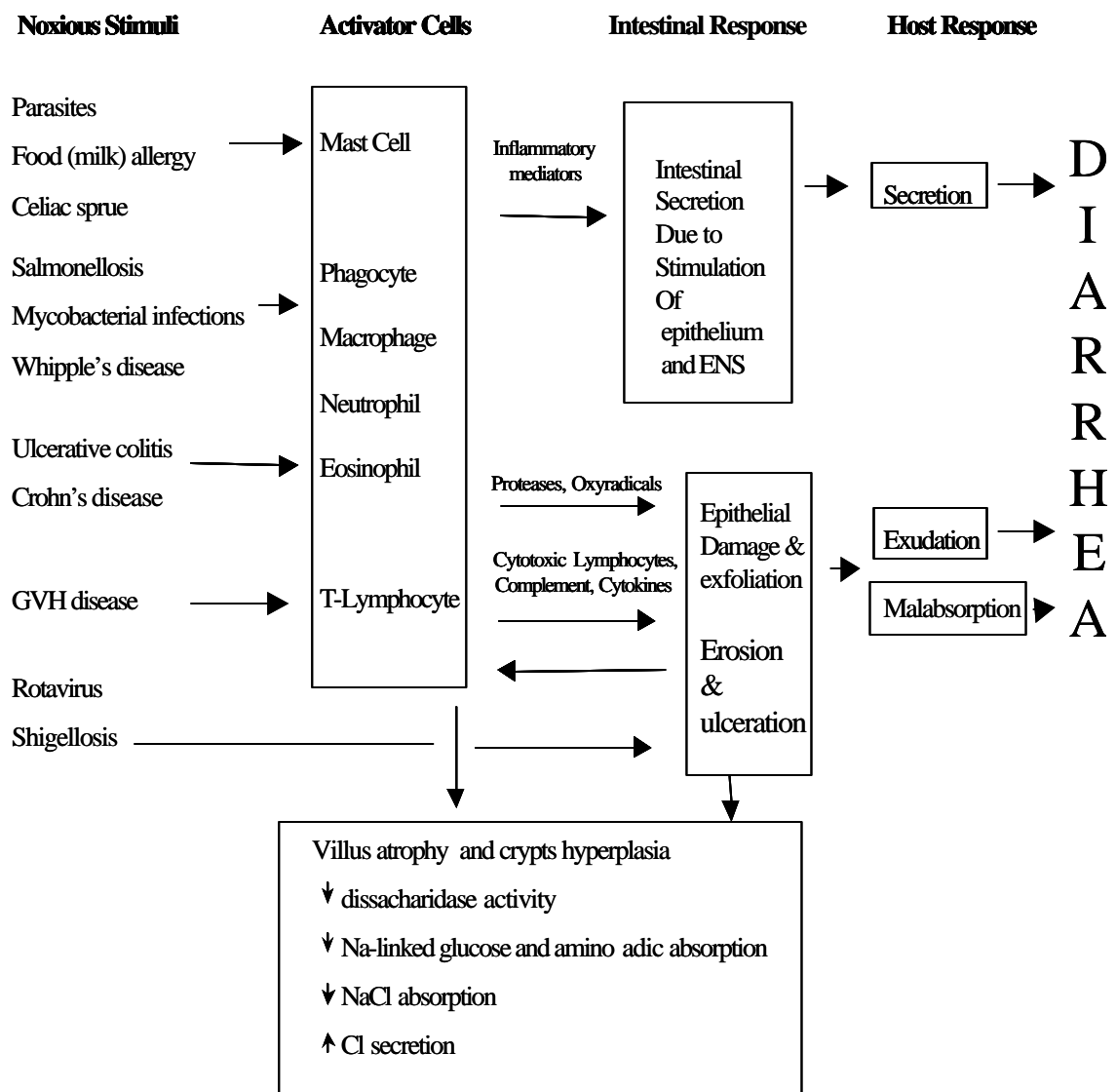
Along with the induction of intestinal secretion, enterocyte damage occurs. There are two general mechanisms of epithelial damage or enterocyte cell death with ulceration: the first is characterized by direct damage and death caused by lumenal or invading microorganisms or parasites; the other involves immunologic mechanisms with damage and death from complement attack, T-lymphocyte-mediated death, or damage and death secondary to proteases and oxidants secreted by mast cells and phagocytes (Figure 3).

Figure 3: Proposed mechanism of cell damage and death. (Yamada, 1999)
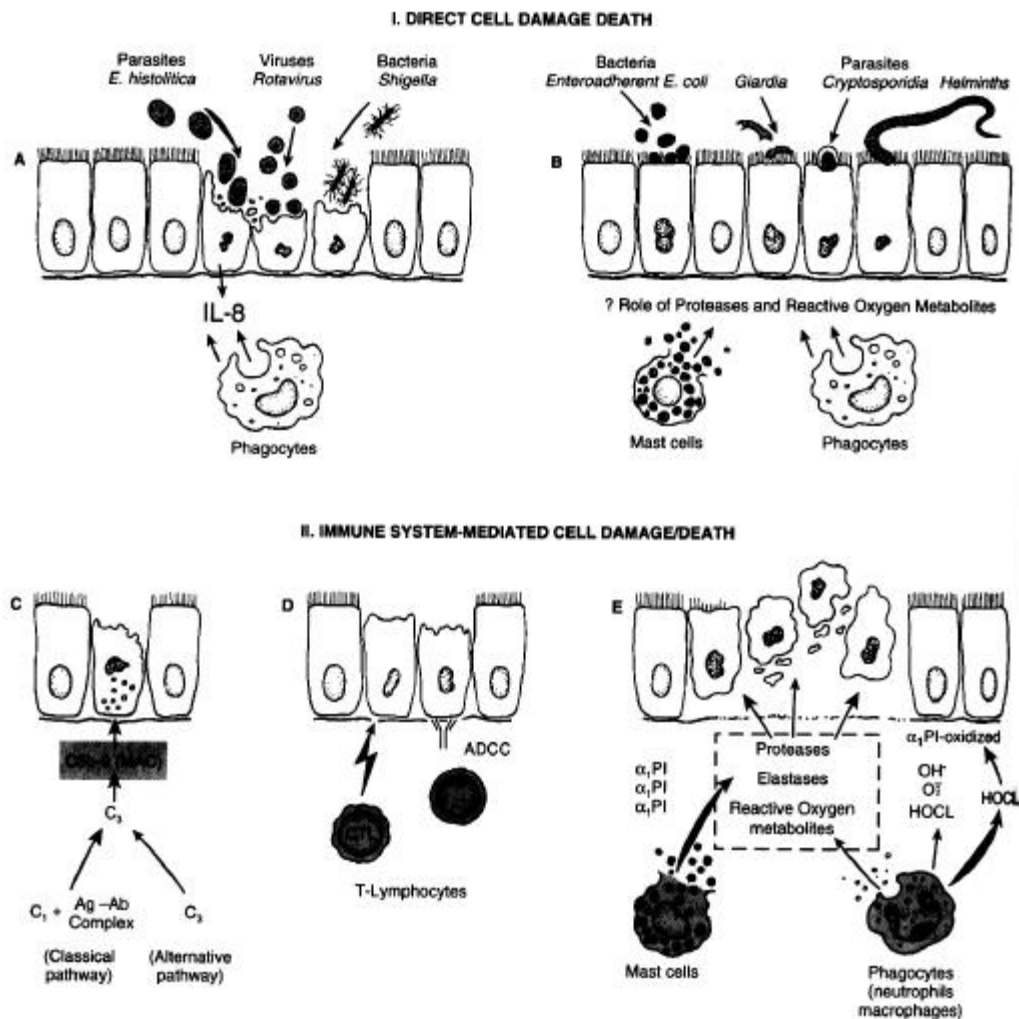
The histologic response to enterocyte damage and death is different in the small intestine and in the colon. In the small intestine, the villi become initially shorter and broader and then disappear completely, while concurrently, there is a compensatory hyperplasia of the crypt [15]. In the colon, the histologic response will produce hyperplastic irregular regenerative crypts with attenuated surface cells. The result of these events is the presence of immature cells on rudimentary or nonexistent villi of the small intestine and on the surface of the colon. These immature absorptive cells have poor disaccharidase and peptide hydrolase activity, reduced or absent Na^+-coupled sugar or amino acid transport mechanisms, and reduced or absent NaCl absorptive transporters. Conversely, the crypt cells and the immature villus of surface cells maintain their ability to secrete CI (and perhaps HCO_3^-). Immune-mediated vascular damage may cause protein to leak from capillaries. If severe ulceration has occurred, exudation from capillaries and lymphatics may contribute to the diarrhea. After damage, epithelial proliferation begins and growth factors, such as hepatocyte growth factor, keratinocyte growth factor, epidermal growth factor, and fibroblast growth factor are released and take part in epithelial repair after ulceration.

T-lymphocyte activation of the immune system is the fundamental cause of villus atrophy and crypt hyperplasia, commonly seen in small intestinal inflammation of various types including infections[16]. Lymphocyte and neutrophil activation also release IL-1 and tumor necrosis factor (TNF) in the blood, and their action on the brain accounts for some of the systemic effects of severe inflammations (fever, malaise, anorexia, and obtundation). These cytokines also activate corticotropin-releasing factor (CRF) in the brain, which stimulates the pituitary-adrenal axis and initiates the glucocorticoid stress response.

Viruses usually are fairly specific for a given species and or certain cells in the host. Unlike bacterial pathogens, most of the viruses do not produce toxins, but rather viruses exert their primary effect directly on the cells that they infect. Viral particles can be detected within surface epithelial cells by electron microscopy and in stool. Although the mucosal damage resulting from viral gastroenteritis has been well documented, insights into disease pathogenesis have been slow in coming[17]. Rotavirus selectively infects and destroys mature enterocytes located in the mid

and upper villous epithelium in the small intestine, without affecting crypt cells. The surface epithelium becomes stunted and flattened. This is followed by a blastic response in the crypts cells, which tends to involve most of the affected villi. During the recovery phase, the enteroblastic cells mature and reconstruct the villous structure. Because of the loss of mature enterocytes on the tips of the villi, the surface area of the intestine is reduced. Diarrhea results from the loss of absorptive function and excess of secretory cells with a net secretion of water and electrolytes, compounded by an osmotic diarrhea from incompletely absorbed nutrients. There is now growing evidence of the role of NSP4, a protein located in the capsid of rotaviruses, acting as an enterotoxin.

1.1.4.2. <u>Nausea and vomiting</u>

Nausea and vomiting are nonspecific symptomatic responses to a variety of noxious stimuli. Nausea is the sensation of an impending urge to vomit, usually perceived in the throat or epigastrium. Vomiting (or emesis) is the forceful ejection of contents of the upper gut from the mouth. Although usually preceded by nausea, vomiting may occur in the absence of nausea in some settings. Retching involves a coordinated action of the striated musculature of the abdomen and thorax, which may precede vomiting, but it involves no discharge of gastric contents from the mouth.

Vomiting results from the coordinated interaction of peripheral and central neural, humoral, somatic, muscular, and gastrointestinal myoelectrical and muscular fenomena and may be initiated by stimuli acting on a variety of anatomic structures within the central nervous system and peripherally. The area postrema on the dorsal surface of the medulla at the caudal aspect of the fourth ventricle is believed to represent the chemoreceptor trigger zone that is responsive to a broad range of neurochemical activators. Peripheral afferent pathways involved in emesis may be activated by stimulation of receptors within the gastrointestinal wall.

1.1.5. DIAGNOSTIC METHODS

There is increasing recognition of the widening array of enteric pathogens associated with illnesses of the gastrointestinal tract. Many of these organisms are easily transmitted through food or water, or from one person to another, and some are devastating to individuals with compromised immune systems or structural abnormalities of the gastrointestinal tract. With the rapid globalization and industrialization of our food supply and with a multiplicity of recognized pathogens and diagnostic tools, the challenge of determining optimal, cost-effective means for appropriate diagnosis and public health control of diarrheal illnesses is great [18].

The diagnosis and treatment of the infectious diarrheas are complicated by the fact that, although these diseases are common, the cost of making the diagnosis and delivering specific (antibiotic) therapy is high, especially for a disease that is usually mild and self-limited.

The lack of specific diagnosis can hinder appropriate management and treatment of many infections. Although the patient's history and clinical findings may provide important clues to likely etiologies, for some pathogens an organism-specific diagnosis is required.

1.1.5.1. Diagnosis of bacterial infections

Because of increasing threats from antimicrobial-resistant infections, side effects of treatment with antimicrobial agents, superinfections when normal flora is eradicated by antimicrobial agents, and the possibility of induction of disease-producing phage by antibiotics, the adequate diagnosis of bacterial infections is crucial. The diagnostic methods can be divided into non-specific and specific tests and are summarized in Table 2.

Stool culture

The gold standard for the diagnosis of acute bacterial gastroenteritis is the stool culture. Although stool cultures are commonly requested, the wide spread use of this test may not be warranted due to the low percentage of gastroenteritis caused by bacterial agents. If one calculates the cost using the yield and price of stool cultures, the cost per positive result can be US$952 to $1200[19]. This

impressive cost is derived from the inability of the test to detect the most likely pathogens. Selective testing can improve the yield and usefulness of stool testing.

Table 2: Diagnostic methods for bacterial enteric pathogens

Nonspecific Tests

Microscopy

Fecal lactoferrin assay

Fecal leukocytes

Stool occult blood

Complete blood count

Routine Screening culture

Enteric agar media

    Differential media (e.g. MAC, EMB)

    Moderately selective media (e.g. Hektoenn enteric,

        xylose-lysine-desoxycholate,Salmonella-Shigella)

Enrichment broth

Sorbitol-MAC agar

Organism-Specific Identification

Organism	Media or method
Salmonella	Brilliant green or bismuth sulfite agar
Campylobacter	Skirrow's formula, Campy-BAP, Butzler's formula, Prenton's formula.
Aeromonas	BAP, MAC, EMB
Vibrio	TCBS agar, V. cholerae 01 antisera
Yersinia	Cefsulodin-irgasan-novobiocin (CIN) agar.
C. difficile	Cycloserine-cefoxitin-fructose-egg yolk (CCFA)
	anaerobically, C. difficile toxin EIA.
Enterohemorragic	
E.coli	Sorbitol MAC, 0157 antisera, H7 antisera

Screening for inflammatory diarrhea: Direct microscopy and lactoferrin testing.

Several groups have suggested that it is more useful to screen first for the relative minority of diarrheal illnesses that are inflammatory or invasive, since there are the most likely to be caused by the invasive pathogens for which culture (*Salmonella, Shigella, Campylobacter, Yersinia)* or toxin testing (toxigenic *C. difficile*) is usually available.

An inflammatory etiology can be suspected on the basis of fever, tenesmus, or bloody stools and can be confirmed by microscopic examination of stool for fecal polymorphonuclear leukocytes or simple immunoassay for the neutrophil marker lactoferrin (Leukotest;TechLab). The disadvantages of microscopy are that the yield is best with fresh-cup specimens and that specimens must be examined by an experienced microscopist. Some studies suggest that testing for fecal lactoferrin may be more sensitive than microscopic examination. Disadvantages of lactoferrin testing include its cost ($3.75 per test kit) and its false-positive results for breast-fed infants. Evidence of an inflammatory response is often not present in noninvasive toxin-mediated infections such as those due to Shiga-toxin producing *E. coli* or enterotoxic *E. coli.*

1.1.5.2. Diagnosis of viral infections

The clinical differentiation of viral gastroenteritis from bacterial gastroenteritis is often difficult. Traditionally, acute gastroenteritis without any demonstrable bacterial pathogen has presumed to be caused by viruses, but it was not until the development of new diagnostic techniques such as electron microscopy (EM)[20], polymerase-chain reaction (PCR)[21,22], immunologic techniques[23,24], latex agglutination, and cell culture that we could identify specific etiologies for this syndrome.

Various epidemiologic factors, such as season and age, as well as clinical manifestations, may be helpful.

Electron microscopy/ Immune electron microscopy (IEM)

Virus diagnosis by EM relies on the detection and identification of viruses on the basis of their characteristic morphology.

EM is the only method that can simultaneously detect and identify each of the important viral agents in the same specimen. Another advantage of the EM is the speed (a clinical specimen can be processed within minutes after collection and in many cases, virus morphology alone is sufficient characteristic to permit family identification). This method has several limitations: it's not possible to examine multiple specimens coincidentally, limiting its use in clinical laboratories, and there must be a minimum number of virus particles present in the specimen in order to be detected. The introduction of IEM, were immune serum is mixed with the virus-containing sample and applied to an electron microscope, has increased the sensitivity of EM.

Enzyme immunoassay (EIA)

This method was first described by Avrameas and Guilbert in 1971[25]. The general principle is that an antigen or antibody bound to a solid phase is used to separate free antibody or antigen from a specimen and the resulting complex is measured by color change in a substrate (Figure 4).

This method offers several advantages: it's fast, has a low cost and it can be easily automated. In general it has good sensitivity and specificity.

There are several commercially available EIA for detection of rotaviruses, adenovirus, and astroviruses.

Figure 4: ELISA for antigen detection

Solid-Phase Surface

1.Capture Antibody (Ab1) Ab1

2. Test Specimen

3. Enzyme-Labeled Detector Antibody (Ab1) --E

4. Substrate ⟹ Color
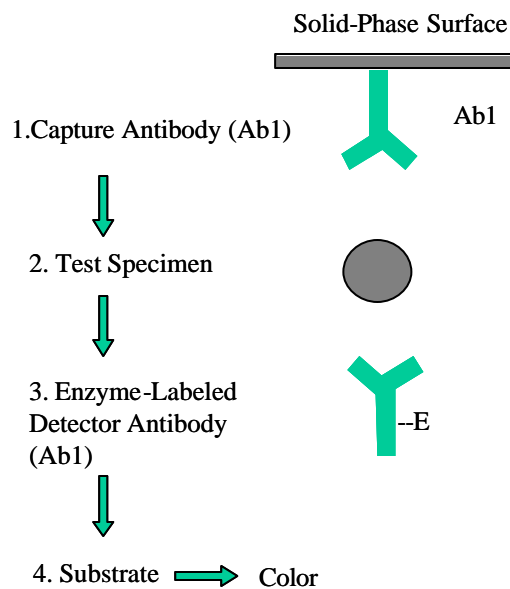
Polymerase chain reaction/ Reverse transcriptase-polymerase-chain reaction (RT-PCR)

PCR was first described by Saiki and Mullis and it is a rapid procedure for in vitro amplification of DNA (or RNA by RT-PCR)[26,27].

This is a procedure based on the ability of DNA polymerase to copy a strand of DNA by elongation of complementary strands initiated from a pair of closely placed oligonucleotide primers. Theoretically, each cycle of the reaction doubles the amount of target DNA, resulting in million fold levels of amplification. As with all the nucleic acid amplification procedures, some information about the target sequence must be available in order to design the two single-stranded DNA oligonucleotide primers that will be used in PCR to amplify target DNA (Figure 5). This technique is highly sensitive and specific given the appropriate design of the primers, and is amenable to automation being broadly used, both in research and clinical laboratories.

Figure 5: The polymerase chain reaction.
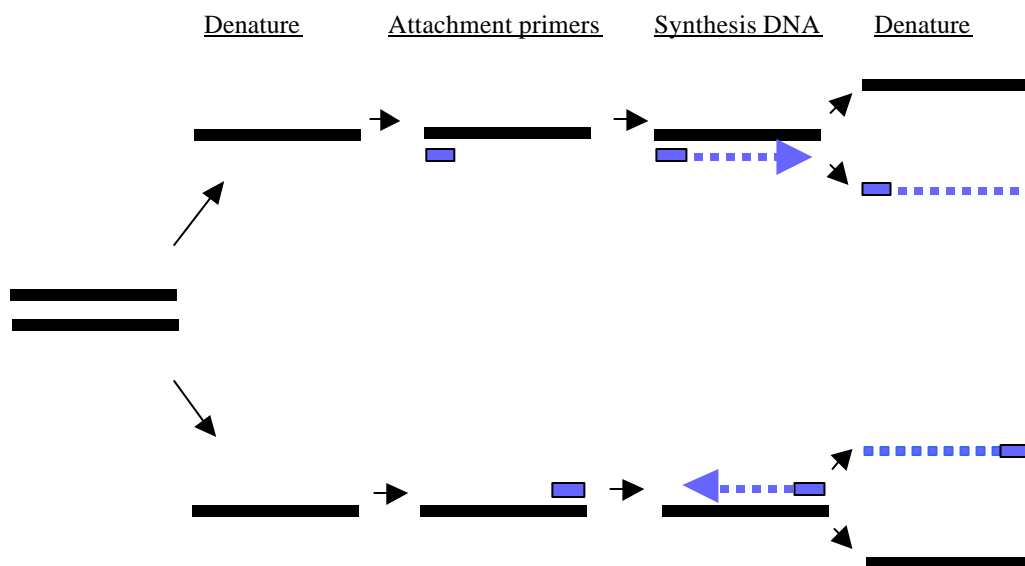
Cell culture

Since 1949, when it was reported that poliovirus multiplied in animal cell culture[28], scientists have been trying to cultivate or detect in vitro those viruses that cause human disease. Viruses can initially be differentiated by (1) the pattern of cytopatic effect, (2) the specific cells in which the cytopatic effect is induced, and (3) the rapidity of the appearance of cytopatic effect.

Cell culture represents the current "gold standard" for the isolation and identification of viruses even though there is sometimes such a lengthy incubation period that results are simply for verification of diagnosis. Isolation is a very sensitive method, because theoretically, a positive result can be obtained with a single infectious virion. Unfortunately, not all viruses are capable of multiplying in vitro (i.e. human caliciviruses), or they multiply in very low titers. Other gastroenteritis-causing agents, like rotavirus, astrovirus and enteric adenovirus can be cultivated in special cells lines.

1.1.6. THERAPY

1.1.6.1. Rehydration

The most common risks with diarrheal illnesses are dehydration and, in developing countries, malnutrition. Thus, the critical initial treatment must include rehydration, which can be accomplished with oral glucose or starch-containing electrolyte solution in the vast majority of cases. Although many patients with mild diarrhea can prevent dehydration by ingesting extra fluids, more severe diarrhea requires specifically formulated oral rehydration solutions or even intravenous solutions. The WHO recommended electrolyte concentration for the oral solutions are Na^+ 90 mM, K^+ 20 mM, $Cl^?$ 80 mM, $HCO_3^{??}$ 30 mM, and glucose 111 mM. The evidence supporting this recommendation for all patients with dehydrating diarrhea is well documented [29-35].

1.1.6.2. Antimicrobials

Because of the increasing threats of antimicrobial-resistant infections, side effects of treatment with antimicrobial agents, superinfections when normal flora is eradicated by antimicrobial agents, and the possibility of induction of disease-

producing phage by antibiotics, such as Shiga-toxin fage induced by quinolone antibiotics [36], any consideration of antimicrobial therapy must be carefully weighed against unintended and potentially harmful consequences.

One situation in which empirical antibiotics are commonly recommended without obtaining a fecal specimen is in cases of traveler's diarrhea, in which bacterial pathogens are likely causes, and prompt treatment can reduce the duration of an illness from 3-5 days to < 1-2 days. Some also considerer empirical treatment of diarrhea that lasts longer than 10-14 days for suspected giardiasis, especially if the patient's history of travel or water exposure is suggestive. Also, for patients with febrile diarrheal illness, especially those believed to have moderate to severe invasive disease, empirical treatment should be considered (after a fecal specimen is obtained).

The decision to treat suspected or documented Shiga-toxin producing *E. coli* with an antimicrobial agent should be considered carefully, as it may worsen the risk of develop hemolytic uremic syndrome.

1.1.6.3. Antidiarrheal agents

Most of the current antidiarrheal agents act by altering the intestinal motility, causing a delay in the microorganism clearance from the bowel, and thus enhancing the chance of invasion and increasing the carriage time.

1.1.6.4. Vaccines

The WHO Programme for Control of Diarrheal Diseases has been heavily involved in promoting and supporting research aimed at the development and evaluation of vaccines against diarrheal diseases. The fact that there is an age-related decrease in diarrheal disease incidence and multiple epidemiological studies also point out that partial protection is provided by natural infection providing hope that successful immunization will be possible.

There are currently several research projects focused in diarrheal diseases and development of vaccines against rotavirus, *Shigella*, cholera, and enterotoxigenic *E. coli* infections.

The most exciting development in vaccine research has been the recruitment of plant science to the field. Several groups of plant biologists have taken up this challenge by looking into the potential of producing recombinant proteins from

transgenic plants so that the food product can be used as a consumable vaccine. Two strategies have been used. One involves the integration into the host plant's chromosome of a microbial gene encoding an antigenic protein. A second approach exploits expression of a desired foreign gene that has been incorporated into the genome of a common plant virus. If appropriate systems can be developed from edible plants expressing antigens in their tissues, these naturally bioencapsulated vaccines could be ingested with subsequent release of antigen as foods were degraded in the human gastrointestinal tract. Contact of the bioencasulated vaccine with the extensive gastrointestinal-associated lymphoid tissue could induce mucosal immunity followed by humoral immunity secondary to the trafficking of lymphoid cells.

1.2. HUMAN CALICIVIRUSES (HUCVS)

1.2.1. HISTORY

The history of calicivirus begins about 70 years ago in California with the description of several outbreaks of a vesicular exanthema disease affecting swine (VES). It was not until the 1970s that the source of these outbreaks was traced to marine animals, the uncooked remains of which were frequently fed to pigs[37]. The agent was named San Miguel sea lion virus type 1. These viruses and others isolated from different animals showed a typical morphology with a cup-shaped depressions in their superficies which lead to the name calicivirus.

The first virus causing diarrhea in humans was discovered in 1972 during the investigation of an outbreak of gastroenteritis at an elementary school in Norwalk, Ohio. After excluding bacterial etiology, stool filtrates were administered to volunteers who also became ill. The study of stool samples from this outbreak and from the volunteers using IEM showed non-enveloped 27-nm virus-like particles: the "Norwalk agent" (NV)[20]. Posterior studies of outbreaks and sporadic cases of gastroenteritis showed morphologically similar agents, referred as "small round structured viruses" (SRSV), that like the "Norwalk agent", could not be amplified in cell culture or in animal models. These viruses had been designated loosely by the location where the strain was detected (e.g. Hawaii, Snow Mountain) and

originally were classified as picornavirus or parvovirus on the bases of its appearance by electron microscopy.

In 1976, a research group led by Madeley and another by Flewett were the first to find morphologically typical caliciviruses of human origin in the stools of children with gastroenteritis[38,39]. Over the next few years, several reports of gastroenteritis associated with viruses presenting the same morphology that those isolated in animals lead to the grouping of all these viruses under the name of "classical human calicivirus". One of the most extensively studied outbreaks was the one occurring in a home for infants in Sapporo (Japan) in 1977[40]. This virus become the prototype strain of the "classical human calicivirus" later renamed as Sapporo-like viruses (SLV).

In the early 1990s, cloning and sequencing of the Norwalk and Southampton viruses revolutionized the study of human calicivirues[41]. Sequencing information permitted assignment to the family *Caliciviridae* of SRSV lacking the cup-shaped depressions previously thought to be characteristics of calicivirus. These viruses were a grouped under the name of Norwalk-like viruses (NLV)

Unlocking the genomic organization of this ill-defined group of viruses led immediately to the development of sensitive molecular diagnostics (e.g. RT-PCR, hybridation probes) that resulted in studies to better understand the epidemiology of these viruses[42,43].

With the new diagnostic techniques, human caliciviruses that had been rarely detected by EM in fecal samples from patients with acute gastroenteritis now has emerged as the most common pathogen identified in outbreaks of gastroenteritis[44-46]. Furthermore, outbreaks that in the past might have been considered small and geographically focal could now be linked globally by molecular epidemiology techniques. Some outbreaks have been traced back to fecally contaminated food and/or water in which the same virus as the outbreak strain could be identified, confirming its causal link and opening the way to screen foods and water for evidence of viral contamination [47-49].

1.2.2. MORPHOLOGY

Caliciviruses are small (27-38nm), non-enveloped viruses with icosahedral symmetry. By electron microscopy two different morphologies have been described: viral particles with cup-shaped depressions in their surface, representing the Sapporo-like genera (Figure 6), and rounded particles in which the depressions are difficult to see, representing Norwalk-like viruses, also known as "small rounded structured viruses" (Figure 7).

Figure 6: Electron microscopy of Sapporo virus

Figure 7: Electron microscopy of Noewalk virus

The capsid is composed of 90 dimers of the major structural protein arranged on a T=3 icosahedral lattice. A characteristic feature of the capsid architecture is the 32 cup-shaped depressions at each of the icosahedral fivefold and threefold axes. The radiographic crystallographic structure of the calicivirus studied in recombinant Norwalk virus capsid reveals a single protein that is organized into two domains: a first domain with a classic eight-stranded [beta]-sandwich motif and a second protruding domain with a novel structure that is connected by a flexible hinge to the shell domain.

Figure 8: Capsid structure of Norwalk virus.

1.2.3. GENETICS

Caliciviruses are genetically diverse but share major features of genomic organization. The genome consists of a linear, positive-sense, single stranded RNA molecule of 7.4 - 8.3 Kb. A protein (VPg) is covalently attached to the 5' end of the genomic RNA and the 3' end is polyadenylated. All caliciviruses have at least three genes. Subgenomic RNA (2.2-2.4 kb) is synthesized intracellularly.

All calicivirus genomes are organized with a large non-structural polyprotein gene, located in open-reading frame 1 (ORF1) that precedes a single structural capsid protein gene. The non-structural polyproteine encodes for a 2C helicase, 3C protease, and 3D RNA-dependent polymerase similar to picornavirus[50]. All caliciviruses have a small 3' terminal ORF encoding for a basic protein (ORF3). It has been speculated that the ORF3 protein may function in viral RNA encapsidation and may aid in regulating assembly of the calicivirus virions[51]. Phylogenetic analyses of sequences from various regions of the genome shows that these viruses can be divided into 4 distinct groups containing two fundamentally different genomic organizations [52,53] (Figure 9). In these two possible configurations, ORF1 is either fused to and contiguous with the capsid ORF, forming a single giant polyprotein running almost the entire length of the genome, or the capsid gene is encoded in a separate reading frame (ORF2) that overlaps the 3' terminus of ORF1 by a few nucleotides. However, in both cases, the major capsid protein is also encoded by a separate subgenomic RNA molecule [54,55].

Replication occurs in the cytoplasm and two major positive-sense RNA species are found in infected cells with animal caliciviruses. The genome-sized positive-sense RNA serves as the template for translation of a large polyprotein that undergoes cleavage by a virus-encoded protease to form the mature nonstructural proteins. A subgenomic-sized positive strand RNA co-terminal with the 3' region of the genome is the template for translation of the major viral capsid protein as well as the 3'-terminal ORF product.
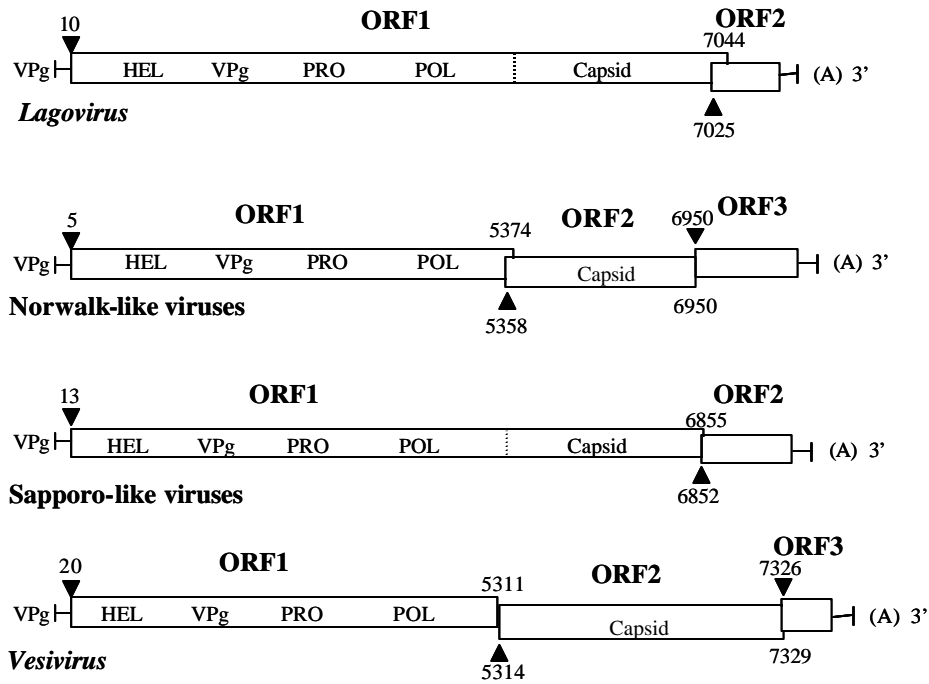
Figure 9: Calicivirus genomic organization[56]

1.2.4. TAXONOMY

Human caliciviruses are included in the family *Caliciviridae*. This new family first appeared in the Third Report of the International Committee on Taxonomy of Viruses (ICTV) in 1979 [57]. Common features for this family included the presence of a single major structural protein from which the capsid is constructed and the appearance of 32 cup-shaped depressions on the surface of the virion. The name of the new family was derived from the Latin word *calyx*, which means cup or chalice.

In the Sixth Report of the ICTV published in 1995, all viruses in the *Caliciviridae* were grouped into 1 genus, *Calicivirus*, although it was apparent at that time that calciviruses were genetically and antigenically diverse and found in a broad range of hosts[58,59].

In 1998 the Calicivirus Study Group presented a proposal for the division of the family *Caliciviridae* into four new genera with one type specie in each genus and to remove the hepatitis E virus from the *Caliciviridae family*. The ICTV approved this proposal being the final taxonomic status of the *Caliciviridae* in the

Seven Report of the ICTV summarized in Table 3. NLVs are further divided into two genogroups based on phylogenetic analyses of both structural (capsid) and nonstructural (polymerase) genes (genogroup I with NV, DSV and Southampton Virus, and genogroup II with Lordsdale, Hawaii, Mexico and Snow Mountain Virus as the main strains). Division of SLVs in three different genotypes with London/92, Sapporo/82, and Houston/90 as a representative strains has also been proposed (Figure 10)

Table 3: Classification of the family Caliciviridae

Genus	Type species
Lagovirus	Rabbit hemorrhagic disease virus
"Norwalk-like viruses" (NLVs)	Norwalk virus
"Sapporo-like viruses" (SLVs)	Sapporo virus
Vesivirus	Vesicular exanthema of swine virus

Figure 10: Relationship between caliciviruses based on phylogenetic analyses in the capsid region (Berke 1999)

26
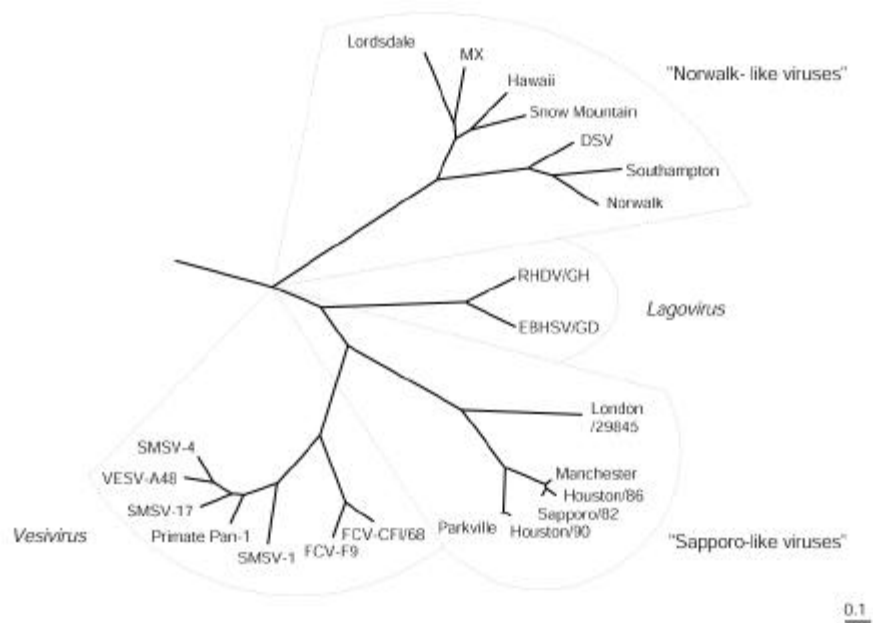
The genera "Sapporo-like viruses" and "Norwalk-like viruses" have been implicated in sporadic and epidemic cases of gastroenteritis in humans and are known as human caliciviruses.

1.2.5. EPIDEMIOLOGY

Environmental surveys find that caliciviruses are ubiquitous and apparently stable in the environment (marine caliciviruses remain viable more than 14 days in 15°C seawater[60]), providing a ready source of virus for potential infection.

HuCV causes outbreaks and sporadic cases of acute gastroenteritis affecting all age groups and different settings (community and institutionalized patients)[46,61] with a worldwide distribution[46,62-69].

The real incidence of HuCV infection is unknown due to the lack of a commercially available test suitable to use in clinical practice. Epidemiological surveys estimate that 95% of outbreaks of selected food-related viral gastroenteritis is the United States are caused by human caliciviruses, representing 23,000,000 episodes per year[11]. Detection of HuCV in sporadic gastroenteritis is highly variable depending on the setting of the study (community, clinic, hospital), the age of the population, and the detection method used: the range goes from 2% in a study conducted by Waters and al [70] using EM up to 20% in a study in Finish children using RT-PCR as a detection method[71].

Calicivirus infection, as measured by antibody prevalence, is nearly universal in children <5 years of age in developing countries[72,73] while in developed countries the acquisition of antibodies is delayed (<20% in the same age group) [64,72,74-77].

Among human calicivirus, NLVs are detected more frequently, though it is not clear if this is due to the fact that NLV infection is more severe than SLV infection, requiring more frequent medical care. Most epidemiological reports describe predominant circulation of strains belonging to genogroup II[78-81]

Epidemiological studies conducted in eight countries reveal that, although NLV were reported throughout the year, NLV-associated gastroenteritis reports

peaked in the winter. This seasonality was present even where outbreaks occurred in institutions or were traced to contaminated food[82].

1.2.6. PATHOGENESIS

Calicivirus infection is transmitted person to person via the faeco-oral route, through fomites, by aerosol spread particularly when a patient vomits, and through contaminated food (shellfish and deli foods mainly) or water (including recreational waters) [83,84]. Because the infectious dose is very low, the attack rate is often high: 30% to 80% in reports of foodborne outbreaks [85] and 30% to 60% in outbreaks that involve person-to-person transmission[86,87]. During foodborne outbreaks, secondary person-to-person transmission is also common.

The incubation period has a mean of 24-48 hours, with a range of 4 to 77 hours. Maximal excretion of virus in feces occurs at the onset of illness and shortly thereafter. The longest detection of virus infection is up two weeks after the onset of illness. Caliciviruses can be detected in stool and vomitus specimens and the amount of excreted virus correlates with the intensity of clinical symptoms.

Pathologic examination of the intestines of adult volunteers infected with Norwalk virus showed abnormal histologic findings in the mucosa of the proximal small bowel, including mucosal inflammation with mononuclear cell infiltration, absorptive cell abnormalities, villous shortening, crypt hypertrophy, and increased epithelial cell mitosis [88]. The gastric and colonic mucosa remains histologically normal. The abnormal findings persist for at least four days after clinical symptoms have ceased, however, the virus has not been detected within abnormal mucosal cells. Histopathologic examination of patients following infection with SLVs remains lacking. Clarification of these pathophysiologic issues may result from the recent detection of NLVs and SLVs infections in domestic animals [89-91].

1.2.7. CLINICAL MANIFESTATIONS

HuCVs are a well known cause of acute gastroenteritis in humans. The severity of this infection is broad: from subclinical infection as shown in experimental studies and in serosurveillances where seroconversions were not always associated with symptoms to severe gastroenteritis requiring hospitalization[58-60].

Common manifestations are diarrhea, vomiting, nausea, abdominal cramps, stomach pain, fever, headache, and malaise. Stool specimens generally do not contain blood or mucus [71,93-95]. The symptoms usually last one to four days and does not lead to chronic infection.

Clinical manifestations of NLV and SLV infection are different: the clinical picture of SLV infection is one of diarrheal illness with little or no vomiting, in sharp contrast to the clinical picture of NLVs where the most prominent symptom is vomiting[96]. Because of the absence of vomiting, SLV strains appear to be uncommon as a cause of severe gastroenteritis [66]. According a study conducted in Japan, NLV infection causes more severe gastroenteritis (comparable to rotavirus) than SLV. Although the severity of gastroenteritis caused by NLV and by RV was similar, RV usually was associated with higher fever than NLV infection. No significant difference was found in the clinical characteristics and severity of acute gastroenteritis regardless of the genetic clades in NLV and SLV[92].

Studies of outbreaks caused by NLVs show that the clinical manifestations differ among adults and children: nausea and stomach pain are the most common symptoms for both adults and children, diarrhea is more common among adults than it is among children, whereas the reverse is true with regard to vomiting. Chills, headache and myalgia are also frequent complaints in adults[61,97].

1.2.8. IMMUNOLOGY

Although several studies have been performed in order to elucidate the immunology of calicivirus infection, a complete understanding of immunity to these viruses remains elusive mainly due to the refractivity of this virus to grow in cell cultures and the lack of animal models . The study of immunity to calicivirus infection has been focused on the Norwalk virus. It is assumed that immunity to other members of the Norwalk-like genogroup is similar. However, we know much less about immunity to SLVs, and it is possible that immunity to these agents differs from that to NV and related virus.

A study published by Dolin demonstrated that most adult volunteers become ill after exposure to the virus, implying either that natural immunity to this virus is not widespread in the general population or that the pathogen easily evades host

immunity[98]. Subsequent studies demonstrated that volunteers did not develop illness if they were re-challenged with the original homologous inoculum between 6 and 14 weeks of the initial exposure, suggesting that short-term immunity to homologous virus develops [88,99].

Long-term immunity was addressed by Parrino *et al* and by Johnson *et al* in homologous re-challenge studies with NV. In Parrino's study, six of the 12 volunteers who were administered the 8FIIa NV inoculum became ill. When all 12 were re-challenged with the identical inoculum 27-42 months later, the 6 who developed symptomatic illness after the initial inoculum became ill once again, suggesting that long-term immunity was not conferred by a single infection with NV. Four of the six who became ill after the second challenge were given a third inoculum 4-8 weeks after the second was administered. Although 1 of the 4 volunteers became ill again, 3 did not. These findings were in agreement with those of earlier studies that suggested short-term immunity (up to 14 weeks) usually follows NV gastroenteritis. Parrino *et al* also found that the level of serum antibody, before initial challenge, could not be used to predict whether an individual would develop illness [100]. These findings were confirmed by Johnson *et al* in a study where volunteers were challenged with NV up to 4 times within intervals of 6 months. This study showed that progressively greater resistance occurs with repeated virus exposure no matter what the previous serological status was, suggesting that host susceptibility to develop illness was not completely explained by previous exposure to the virus (intrinsic host factors)[101]. To study the determinants of resistance to NV infection in adult volunteers, Greenberg *et al* analyzed serum and local jejunal antibody levels in volunteers before they received the NV inoculum. No correlation between these antibody levels and resistance to infection could be demonstrated. On the contrary, the geometric mean titer of Norwalk antibody in jejunal fluid of susceptible volunteers was significantly higher that that in resistant volunteers. A similar trend was noted for serum antibodies, suggesting that nonimmune mechanisms may play a role in determining response to NV challenge[102].

Lew *et al* concluded in a study published in 1994 that low preexisting NV IgG titer in children was associated with susceptibility to developing NV infections[74].

By contrast, adults with preexisting antibody to NV were not protected against infections in neither the volunteer study or in natural outbreaks[93,103,104]. The apparent incongruity between the findings in children and those in adults may be explained in part if it is accepted that the direct correlation between serum antibody level and protection observed in young children reflects short-term, recent exposure. In adults in developed countries, serum antibody level is probably an indicator of past exposure rather than recent infection. The frequency of exposure to NV in children of certain populations may also influence the development of immunity to the virus, since exposure may be so frequent that long-term immunity is not required.

Unlike rotaviruses, enteric adenoviruses and astroviruses, human caliciviruses cannot be cultivated in vitro, so antigenic relationships have been determined mostly by binding of antibody (antigenic type) rather than neutralization (serotype). Strains in different genera are antigenically distinct. Strains in different species are antigenically distinct in antigen-detection ELISAs, but when acute and convalescent serum pairs are tested in antibody-detection assays, responses across species occur. Madore *et al* reexamined the antigenic relatedness among different NLVs (NV, SMV, and HV). Most volunteers (75%-90%) who were challenged with one of these viruses showed elevated serum antibody titer to the homologous challenge virus. Serum antibody titer to heterologous viruses also rose in those who had had a significant seroresponses to the homologous virus. The magnitude of the seroresponse to homologous virus was substantially greater that to heterologous virus, as observed previously[105]. These findings indicate that, at least within genera, some common epitopes exist. A general trend has been observed using a combination of antigen- and antibody-detection assays: strains positive in an antigen-detection assay share >95% sequence identity with each other in the partial RNA polymerase gene of the CV genome. Strains inducing antibody responses detected in an antibody-detection assay share >85% sequence identity in the same genomic region[106]. This pattern has been complicated by recognition of naturally occurring recombinants[107].

1.2.9. DIAGNOSTIC METHODS

More than 30 years after the discovery of human calicivirus, the detection of this virus in clinical specimens is still a challenge.

The history of the detection of this human calicivirus begins with the discovery of Norwalk virus using IEM [20]. Because this virus could not be cultivated, a number of techniques based on reagents obtained from patients or experimentally infected volunteers were developed, including radioimmune assays (RIAs)[108,109], blocking RIAs [110], ELISA [111,112], and immune adherence hemagglutination assays [108]. A western blot technique to detect HuCV proteins from fecal specimens was developed to detect viral antigens and antibodies [113]. These techniques greatly advanced the knowledge of HuCVs and associated illness, however, because of the limited supply of reagents from humans, these techniques were used only in a few research laboratories. Furthermore, it was difficult to determine the infection history of volunteers who were administered calicivirus before providing serum and stools as reagents, making the specificity of assays using these reagents uncertain.

The cloning of the prototype NV and many related strains allowed the development of new diagnostic assays [50,114]. Determination of the nucleic acid sequence of different calicivirus strains allowed the design of primers to detect viral RNA by a reverse transcription-polymerase chain reaction [43,78,115-120].

Molecular engineering techniques also permitted the development of diagnostic assays based upon expression of caliciviral capsid antigens [121-126]. The baculovirus-expressed HuCV capsid proteins self-assemble into virus-like particles (VLPs) with similar morphology and immunology to authentic virions but not including the virus' genetic material, providing excellent reagent for use in developing immunologic methods[121].

The genetic and antigenic diversity among the human caliciviruses is a major research challenge. So far the only technique capable of detecting all caliciviruses is EM, but the low sensitivity of this method when the number of viral particles is low and the impracticality of using this technique in the clinical practice make this method sub optimal.

The development of effective diagnostic tests such as a broadly reactive RT-PCR or an immunoassay that recognizes all circulating strains is one of the current topics under more active investigation. The identification of a permissive cell culture system for HuCVs remains an important goal. Without this system it will be impossible to define serotypes by neutralization or to assess the role of neutralizing antibodies in immunity. The ability to quantitate virus by titration in cell culture would facilitate environmental studies designed to assess risk factors for gastroenteritis resulting from exposure to contaminated food or water in which the viability of virus detected by RT-PCR is not known.

2. <u>HYPOTHESIS AND OBJECTIVES</u>

Human calicivirus are an important cause of gastroenteritis outbreaks but the role of this virus as a cause of sporadic gastroenteritis is often considered irrelevant, partly because of the lack of epidemiological data, partly because of the believed that calicivirus causes only mild disease.

The hypothesis of this thesis is that human calicivirus are a significant cause of severe disease in children.

Identification and characterization of viruses causing severe gastroenteritis and to gain a clear understanding of their epidemiology and immunology are necessary in order to prevent their transmission and to develop appropriate prevention strategies. To advance these goals, active surveillance was conducted in the United States to assess the impact of different viruses causing acute gastroenteritis in children and resulting in hospitalization at three sites over a two-year period.

The primary aim of this study is:

1. To assess the prevalence and clinical characteristics of HuCV infection among children resulting in hospitalization during a multicenter study in the U.S.A.

Secondary aims are:

1. To determine the genetic variability of the HuCV strains causing these illnesses, according to geographic and temporal differences.
2. To determine the direct medical costs of these severe HuCV illnesses.

3. <u>MATERIAL AND METHODS</u>

3.1. <u>RESEARCH DESIGN</u>

This thesis is based on the specimens and clinical information collected during a multicenter hospital-based study of rotavirus infection conducted by Dr. David O. Matson entitled, "Impact of rotavirus infection resulting in hospital care in the United States before mass immunization".

3.2. <u>PATIENTS</u>

Patients were enrolled in a longitudinal, prospective manner in three pediatric hospitals: Children's Hospital Medical Center in Cincinnati, Ohio; Children's Hospital of The King's Daughters in Norfolk, Virginia; and Children's Hospital Oakland in Oakland, California.

Children two weeks to 5 years of age admitted Sunday through Thursday in any of the three participating hospitals were monitored during the study period (November 1997 to December 1999). Cases were identified by reviewing admission logs of the hospital.

INCLUSION AND EXCLUSION CRITERIA

a) <u>Inclusion criteria</u>
- The child was 14 days to 5 years of age.
- The child was an inpatient or was being observed in a short-stay unit of the hospital.
- The child had diarrhea, vomiting, or fever.
- The child's illness was less than 7 days duration.
- Written consent was obtained from the child's parent or legal guardian.

b) <u>Exclusion criteria</u>
- If the child was vomiting (no diarrhea) and the child had a respiratory illness that could explain the vomiting.
- If the child had fever (no diarrhea and vomiting) and the fever was attributed to a specific cause (otitis media was not considered a sufficient specific cause to exclude the child) or the child had an immunodeficiency syndrome.

Research personnel reviewed the chart, completed an eligibility questionnaire (Form A), obtained identifying information to facilitate follow-up (Form B), obtained consent (Form C), solicited details about the illness, birth history, underlying health information, and cost of current illness before admission (Form D). Stool specimens were tested for rotavirus by EIA (Form E). Acute blood specimens already collected for diagnostic purposes were identified in the laboratory and retained for the study (form F). The rotavirus results were telephoned to the parents a week after stool collection. During that telephone conversation, details about the non-medical cost of the illness during the hospital stay and the medical and non-medical costs after the hospital stay were solicited (Form G). The information was then transcribed to a database (MS Access 97).

In addition, the hospital information services department provided a data file that contained the patients medical record number, date of birth, date of admission, date of discharge, gender, race, insurance provider, zip code of residence, admission diagnoses, discharge diagnoses, and inpatient charges for all the enrolled patients.

<u>SPECIMENS</u>

Stool samples were collected within the first 24 hours after admission. The specimens were collected in three different forms: in a collection cup, on a diaper, or as a rectal swab. Stool specimens were tested for rotavirus at the site of patient enrollment using a commercially available EIA (Meridian Diagnostics, Cincinnati, Ohio), then sent to the Center for Pediatric Research (Norfolk, Viriginia), and stored at –70ºC until being tested for HuCV.

<u>DEFINITIONS</u>

- **Diarrhea**: For enrollment purposes, diarrhea was defined as an episode of ? 3 stools in a 24-hour period judged by the parent to be looser than normal. Once the patient was enrolled, diarrhea was defined as any number of stools per day considered as a looser than normal by the parents.
- **Vomiting**: The forceful expulsion of gastric contents. At least one occurrence in a 24-hour period constituted an episode of vomiting.
- **Fever**: An axillary or rectal temperature of greater than 38ºC (100.4º F).

- **Gastroenteritis** was defined as (1) diarrhea with an additional symptom, (2) vomiting not related to respiratory illness or an underlying disease, (3) three or more loose stools in 24 hours. Additional symptoms included diarrhea, vomiting and fever.

3.3. METHODS

3.3.1. TO ASSESS THE PREVALENCE AND CLINICAL CHARACTERISTICS OF HUCV INFECTIONS AMONG CHILDREN WITH SEVERE GASTROINTESTINAL SYMPTOMS ENROLLED DURING THE MULTICENTER STUDY.

In order to assess the prevalence of calicivirus infection in the study population, stool samples were tested by RT-PCR for the presence of human calicivirus. The performance of RT-PCR requires three steps:

a) Preparation of the samples and extraction of viral RNA

b) RT-PCR

c) Visualization of the RT-PCR products

a) Preparation of the samples and extraction of viral RNA

General description

The viral RNA was extracted using Trizol Reagent (GibcoBRL). Trizol reagent is a mono-phasic solution of phenol and guanidine isothiocyanate and is an improvement to the single-step RNA isolation method developed by Chomczynski and Sachhi[127]. During sample homogenization or lysis, Trizol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol.

Application to the study samples

A 20% stool suspension in tris-buffered saline was prepared and stored at 4ºC until the extraction was performed.

According to the manufacturer's instructions, 500 µL of 20% stool suspension was centrifuged at 10,000 rpm for 10 min and 400 µL of the upper phase transferred to a fresh tube containing 1000 µL of Trizol reagent (GibcoBRL). After shaking well and incubation at room temperature for 5 min, 200 µL of chloroform was added. This mixture was then centrifuged for 15 minutes at 12,000 rpm and the aqueous phase transferred to a new tube. RNA was precipitated by adding isopropyl alcohol and incubating at $-20º\,C$ for one hour. A positive control (tissue culture of primate calicivirus) and a negative control (water) were included in each round of extractions (22 samples per round) in order to control the quality of the extraction.

The RNA extracted was diluted in water containing 0.4 U/? L of RNasin and stored at -70ºC.

b) RT-PCR

General description

The thermostable polymerase used in the basic PCR process requires a DNA template. In order to apply PCR methodology to the study of RNA viruses like caliciviruses, the RNA samples must first be reverse transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase. This process is called reverse transcription (RT). For the reverse transcription step, avian myeloblastosis virus reverse transcriptase is used to produce a DNA copy of the RNA template using a sequence-specific primer.

The theoretical basis of PCR is analogous to the process of biological amplification, with enzymatic duplication of specific nucleic acid sequences. Using primers oriented with their 3' end facing each other to hybridize opposite strands of the DNA, the DNA polymerase (which catalyzes growth of new strands 5' ✍ 3') will synthesize a new DNA strand by elongation of the primer sequences. One round of synthesis results in new strands of indeterminate length, which, like the parental strands, can hybridize to primers upon denaturation and annealing. These

products accumulate only arithmetically with each subsequent cycle of denaturation, annealing to primers, and synthesis. However, the second cycle of denaturation, annealing, and synthesis produces two single-stranded products that together compose a discrete double-stranded product, which is exactly the length between the primer ends. Each strand of this discrete product is complementary to one of the two primers and can therefore participate as a template in subsequent cycles. The amount of product doubles with every subsequent cycle of synthesis, denaturation, and annealing, accumulating exponentially.

In PCR, three nucleic acids segments (the segment of double-stranded cDNA to be amplified and two single-stranded oligonucleotide primers flanking it?, a DNA polymerase (*T. aq*), deoxyribonucleotide triphosphates (dNTPs), a buffer, and salts are mixed and placed in a thermocycler to proceed to the denaturation, annealing and synthesis steps.

The selection of primers, ion concentration in the buffers, amount of RNA used as a template, enzymes, and temperature at which each step is performed are crucial factors that will determine the sensitivity and specificity of the test.

Application to the study samples

In our study all the samples were tested using the primer pair 289/290. This primer was developed by Jiang [119] and was designed for detection of both, Norwalk and Sapporo-like viruses. This primer targets a conserved region in the polymerase gene resulting in an amplicon of 319 base pairs (bp) and 331 bp for the NLV and the SLV respectively.

Although this primer pair has a broad detection range it is known that there are some strains that are not detected (actually none of the primers published to date detect all the calicivirus strains circulating). The selection of a second or even third primer pair can be done using the available information regarding the current epidemiology of circulating HuCV strains in a specific geographic location and then selecting a primer that target them, or using a multiple primers with a broad spectrum. Because the aim of our study was to assess the epidemiology of HuCV infection, we decided to follow the last approach.

A subset of 200 samples negative by primers 289/290 stratified by site, date of admission and age were tested with two different primers sets (p289a/290a and 289hi/290hijk), both developed by Jiang to detect NLV and SLV with a supposed broader spectrum than 289/290 (data not published). This new primers are variations of 289/290, with the same target region and therefore the expected size of the product is the same that for 289/290. In 289hi/290hijk, four different positive-sense and 2 negative-sense primers have been combined. Because the detection rate in our study was higher with the p289hi/290hijk, all the samples negative with the p289/290 were tested with p289hi/290hijk.

The ion concentration, amount of RNA used as a template, and temperature was optimized for detection of HuCVs.

For the RT step, a 25 µL mixture containing 2.5 µL of 10xPCR buffer (100 mM TrisHCl, 20 mM MgCl$_2$, 500 mM KCl), 2µL of 5 mM dNTP, 1µL of 0.1 µg/µL of negative-strand primer, 0.1 µL of RNasin (40 U/mL), 0.?µL of AMV-RT (20 U/µL), 2.5µL of 1% bovine serum albumin, 13.7 µL of molecular biology grade water and 3 µL of RNA was incubated for one hour at 42ºC.

For the PCR, 25 µL of reaction mixture containing 2.5 µL of 10xPCR buffer, 1 µL of positive-strand primer (0.?µL/µL), 2 µL of *T. aq* polymerase and 19.5 µL of molecular biology grade water was added to the RT reaction mix, overlain with mineral oil and placed into a thermocycler. The amplification cycle program included a denaturation step for 3 min at 94ºC and 40 cycles of denaturation for 1 min, primer annealing for 90 s at 49ºC and primer extension for 1 min at 72ºC. A final extension step was performed for 15 min at 72ºC.

In order to avoid extra manipulation of the reagents, to minimize risk of contamination, and to reduce variability due to pippeting, a master mix for the RT and for the PCR containing all the reagents except the enzymes and the RNA was prepared in a sterile hood, aliquoted in tubes containing the amount necessary for 60 samples, and stored at –70ºC.

c) Detection of the amplified RT-PCR products

General description

DNA is a negative charged molecule that will migrate within an electrical field. The speed at which DNA migrates in a gel depends on the size of the molecule allowing us to differentiate the size of the different PCR amplicons.

Application to the study samples

RT-PCR products were loaded in a 1% agarose gel dyed with ethidium bromide. Electrophoresis was performed in 0.5xTBE buffer for 1 hour at 175 volts. At least one positive control and two lanes with a 1 Kb marker were included in each run (Figure 12). The DNA bands were visualized with illumination by UV light. A picture of the gel was taken using a digital camera and analyzed with image analyses software.

The positives by RT-PCR were confirmed by sequencing (the protocol is described in detail in the next section).

Figure 11: Picture of gel electrophoresis of RT-PCR products by p289hi/290hijk. Track 1, 21, and 22, 1Kb marker; track 12, positive control.
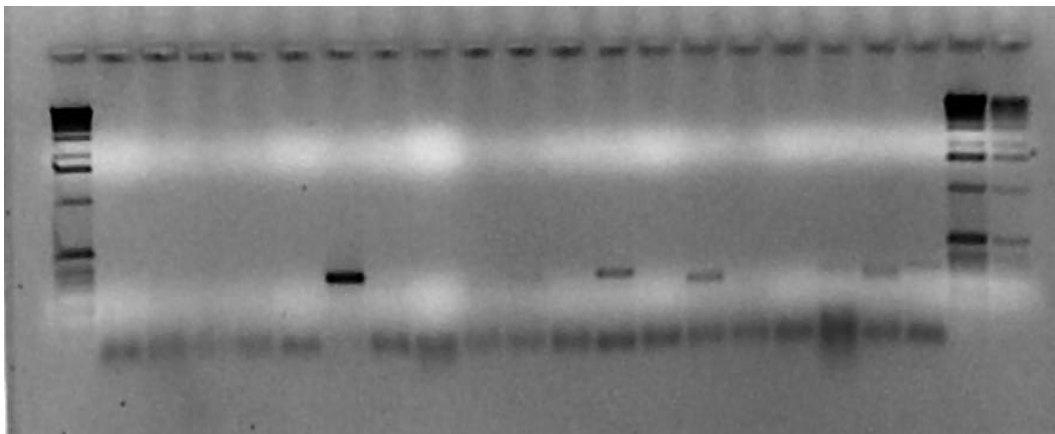
The process of detection and confirmation of positives is summarized in Figure 13. When sequence confirmation of the original PCR product was unsuccessful, a seminested-PCR using p289 and a primer based upon conserved

regions observed in previous sequences from our study (EP1, 5'-TCC CCT CTG GGA TGC CAT GCA C-3' located in nucleotides 4653 to 4674 of the Norwalk virus genome) was performed and sequencing of this new amplicon was attempted.

The clinical and demographical characteristics of the infected patients were analyzed. We developed a score system based on 20-point score proposed by Kapikian (personal communication) and validated by Ruuska and Vesikari[128] to assess the clinical severity at the time of admission of the patients with acute gastroenteritis (Table 4).

Figure 12: Flowchart for the detection/confirmation of calicivirus positive samples
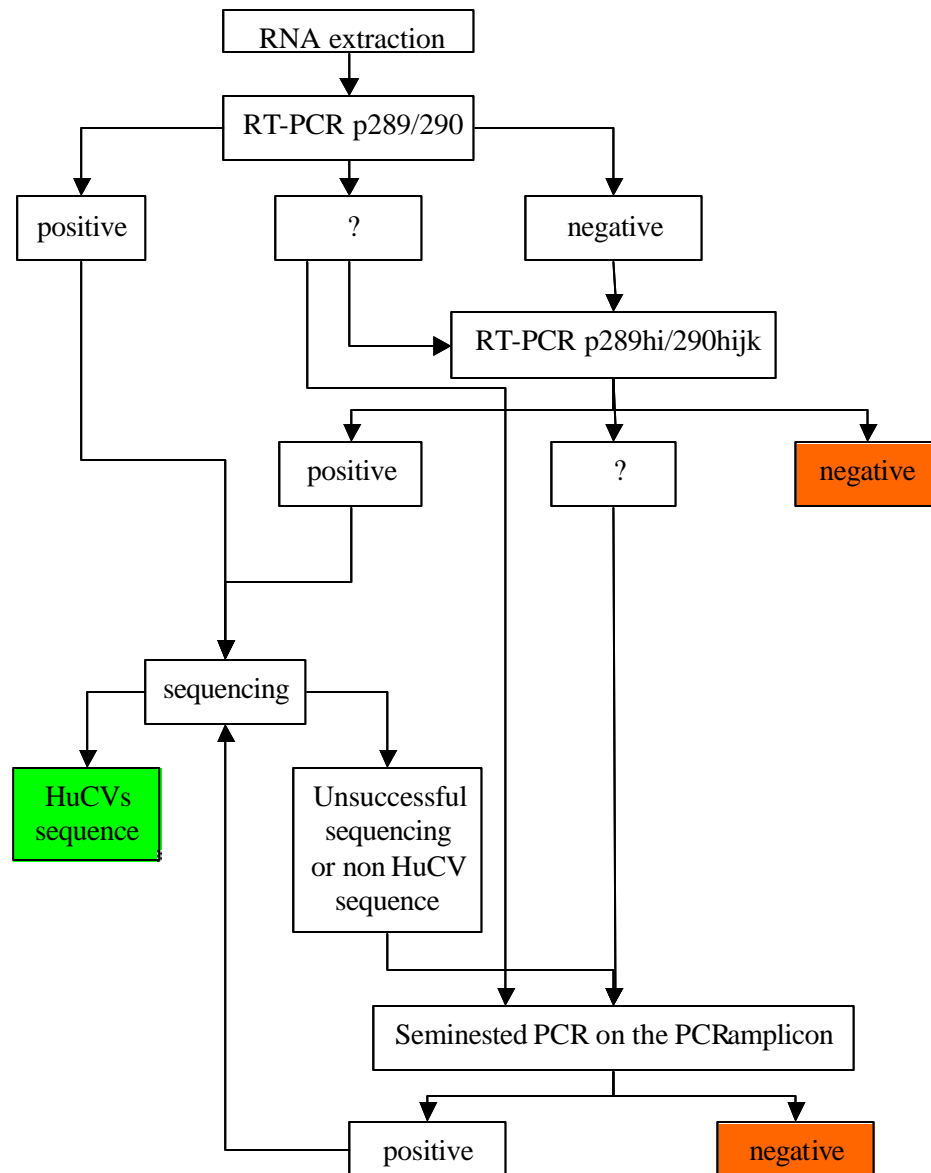
Table 4: Numerical scoring systems for severity of gastroenteritis

Symptom or sign	Vesikari' score (20 points)	Modified Vesikari (17 points)
Duration of diarrhea (days)		
1-4	1	1
5	2	2
>5	3	3
Max no. diarrheal stools/24h		
1-3	1	1
4-5	2	2
>5	3	3
Duration of vomiting (days)		
1	1	1
2	2	2
>2	3	3
Max. no. vomiting episodes/24h		
0	1	1
1	2	2
2-4	3	3
>4		
Fever		
<37.1°C		
<37.1-38.4°C	1	1
38.5-38.9°C	2	2
>38.9°C	3	3
Dehydration		
1-5%	2	
>5%	3	
Treatment		
None	0	0
Rehydration	1	1
Hospitalization	2	2

3.3.2. TO DETERMINE THE GENETIC VARIABILITY OF HUCV STRAINS

In order to determine the genetic variability of calicivirus in our study population, the amplicon generated by RT-PCR in the polymerase region was sequenced.

This process requires four major steps:

a) Cloning

b) Purification of the plasmid DNA

c) Sequencing

d) Analysis of sequences

a) Cloning

General description

Insertion of the PCR amplicon into a vector system (plasmid DNA) and posterior transformation of competent cells with this vector is a quick and effective way to obtain high concentration of an specific amplicon. This process will also allow us to use a unique fluorescent labeled primer pair complementary to the vector for the sequencing reaction.

In the first step, the RT-PCR product is ligated into a vector (i.e. pGEM-T vector) and then the vector with the inserted amplicon is introduced into competent cells (cells with a very permeable membrane) using a thermal process. The pGEM-T vector is a circular plasmid DNA that contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning site within the ? -peptide coding region of the enzyme ? -galactosidase. Insertional inactivation of the ? ?peptide allows recombinant clones to be directly identified by color screening on indicator plates. This vector also contains a ? -lactamase coding region that confers ampicillin resistance to the JM109 competent cells when they have been effectively transformed.

Application to the study samples

The RT-PCR products were cloned using the pGEM-T vector system I (Promega). Following the manufacture's protocol, a mix containing 2?L of the RT-PCR product, 5 ? L of buffer, 1 ? L of DNA ligase and 1 ? L of pGEM-T vector was incubated at 4ºC overnight. Fifty microliters of competent cells (JM109 cells) were transformed by keeping them on ice with the ligation product for 20 min and then heat shocking at 42ºC for 45 sec. The cells were then seeded onto agar plates containing ampicillin, X-gal, and IPTG and incubated at 37ºC for 18 hours.

Recombinants obtained from cloning (white colonies) were screened by PCR using forward and reverse primers targeting the SP6 and T7 polymerase promoters. The expected size of the amplicon is 558-571bp.

b) Purification of plasmid DNA

In order to sequence the amplicon obtained in the RT-PCR, the plasmid DNA from the positive clones has to be purified. We employed a commercially available kit, QIA prep Spin Miniprep Kit (Qiagen), to achieve it. The QIA miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of a high concentration of salt and then washing and elution of the plasmid DNA.

c) Sequencing

General description

The method used for sequencing was the dideoxy chain termination method (also known as Sanger's or the enzymatic method).

The chain termination method involves the in vitro synthesis of a DNA strand by a modified bacteriophage T7 DNA polymerase primed by a synthetic fluorescent-labeled oligonucleotide. In the presence of dideoxynucleotides (ddNTP), the synthesis of the new DNA strand is terminated whenever a ddNTP is added to the growing end of the chain because the dideoxyribose residue lacks both a 2' and a 3'-OH group, and therefore cannot serve as a substrate for the addition

of another nucleotide by DNA. Four separate reactions are set up for each template to be sequenced, each containing one nucleotide as the ddNTP plus all four deoxynucleotides (dNTPs). For each reaction, enzyme catalyzed synthesis will be terminated in a fraction of the population of chains at each site where the ddNTPs is incorporated. The products are size separated by a high resolution denaturing polyacrylamide gel electrophoresis. During electrophoresis, the fluorescently labeled fragments in each lane migrate downwards through the gel. A fixed laser beam excites the fluorescent labeled DNA bands and the light emitted is detected by photodetectors located behind the gel. The photodetector signals are collected, digitized and sent to the computer for storage and processing.

Application to the study samples

Nucleotide sequencing of the RT-PCR amplicons was performed using SequiTherm EXCEL II Long-Read DNA Sequencing Kit-ALF (Epicentre technologies) on an automated sequencer (ALFexpress DNA sequencer, Amersham Pharmacia). The primer used targeted the SP6 polymerase promoter of the plasmid where the RT-PCR products were inserted.

d) <u>Analysis of sequences</u>

General description

Several methods have been developed in order to assess the diversity and the relationship among molecular sequences of different strains from organisms causing disease. The epidemiological importance of tracing strains causing disease in different locations or large outbreaks is well known. Also, the study of differences and similarities between strains of a specific organism can lead us to determine a common ancestor for them, and to hypothesize the evolutionary path for the current diversity. All the information needed for this assessment is stored in the genome.

Application to the study samples

The raw DNA sequences obtained in ALFwin were imported into a local sequence database maintained in a sequence analysis environment OMIGA 2.0, a sequence analysis environment. Each sequence was verified by identifying the the RT-PCR primers and plasmid sequence at both ends and localizing. When necessary, the reverse complement was obtained. The search for missing and/or extra nucleotides was performed by translation to amino acid sequence and comparison to reference sequences. If the reading showed a high number of ambiguities, the DNA was re-sequenced and consensus sequence for the two readings was obtained.

Sequences were compared and aligned with sequences held in the NCBI public databases using BLAST (Basic Local Alignment Search Tool). Multiple alignment at the nucleotide level was performed within OMIGA using the ClustalW (v.1.6) algorithm, then exported to GenDoc (Multiple Sequence Alignment Editor & Shading Utility v. 2.5) and translated to amino acid sequences in order to confirm alignment of homologous regions. Phylogenetic distances were calculated using by Kimura 2-parameter for the major calicivirus clusters. Sequences were summited for phylogenetic analysis along with sequences of representative strains for each genogroup using the maximum likelihood algorithm (DNAML, PHYLIP 3.5). This analysis was performed separately for NLVs and SLVs, and among the NLVs a first phylogenetic analysis was performed grouping sequences obtained from each site. A selection of sequences significantly distinct or representing a distinct cluster from each site was then aligned and analyzed. The phylograms were drawn using TreeView. Branch points of the resulted phylogenetic trees had a confidence level of p<.05.

3.3.3. TO DETERMINE THE DIRECT MEDICAL COSTS OF SEVERE HUCV ILLNESSES

During the enrollment period, an extensive questionnaire was completed for each participant. Based on the information collected, the direct medical cost was estimated for the pre-hospitalization, hospitalization, and post-hospitalization periods.

The components of the direct medical costs analyzed in this study were: visits to a health care provider, tests ordered, medications prescribed and the need for intravenous fluids, and hospitalization costs.

The cost of visits to a health care provider, intravenous fluids prescribed, and tests ordered was assessed for each enrolled patient in the pre and post-hospitalization periods using the parents report and published data on the Medical Fees in the United States 1999 (Practice Management Information Corporation, ISBN 1-57006-129-4).

The cost of the medications was calculated using the parenting report for the type of medication and the prices available in a national web site (www.drugstore.com) for the cost. When several presentations of the drug were available, the most probable to be prescribed was chosen (e.g. suspension versus tablets) and the size was calculated using the patient age assuming a maximum length of therapy of 7 days. Chronic medications were not included in the analysis even it they were prescribed in one of the previous visits due to current illness. If the same medication was prescribed in more than one visit within the pre or the post-hospitalization periods, that medication was counted just once. The cost was adjusted to 1999 US dollars using the medical component of the Consumer Product Index (Bureau of labor and statistics)

For the hospitalization period, the hospital charges were gathered from the hospital databases. These costs were adjusted to the national average using a geographic adjustment factor and to 1999 US dollars using the medical care component of the Consumer Product Index for each year (Bureau of labor and statistics).

STATISTICAL METHODS

All data that were collected during the study period were entered and maintained in a MS Access 2000 database that was designed specifically for this study. The data were transferred into SAS Version 8.01 for statistical analysis.

Exploratory data analyses were conducted through frequencies and normality tests of the data. Chi-square or Fisher's Exact tests were used to test for significant differences between groups of categorical data. T-tests and ANOVA were used to compare the means between groups of normally distributed continuous data, while the Wilcoxon rank-sum or Krusal-Wallis tests were used to compare medians of continuous data without a normal distribution. Linear correlation between variables was examined using Spearman's rank coefficient.

The medical costs associated with a calicivirus infection was explored. MS Access 2000 was used to conduct part of the cost analysis using different queries and modules in order to assign a dollar value to each component. Pre-hospitalization costs included any expenses associated with physician visits, medical tests given or procedures performed, and prescribed medications. Hospitalization costs included all charges incurred during the hospital stay. Actual hospitalization charges were adjusted for the geographical differences in each site by multiplying them by the 1999 geographic factor. Post-hospitalization costs included any charges associated with physician follow-up visits, additional medical tests given or procedures performed, and prescribed medications. The total cost associated with a calicivirus infection was computed by adding all the pre-, post-, and adjusted hospitalization charges. The Wilcoxon rank-sum test was used to compare the median medical costs for different groups of enrolled patients.

4. <u>RESULTS</u>

4.1. EPIDEMIOLOGY AND CLINICAL CHARACTERISTICS OF HUMAN CALICIVIRUS INFECTION

4.1.1. DEMOGRAPHICAL CHARACTERISTICS OF THE STUDY POPULATION

a) Patients admitted during the study period

The total number of patients admitted to any of the participating hospitals during the study period with ages in the range of inclusion to the study was 45,334. The distribution of admissions during the study period among the three sites was as follows (Figure 13)

Figure 13: Study population

Patients admitted during the study period=85,727		
Cincinnati=26,721	Oakland=44,467	Norfolk=14,539

53%

Patients admitted during the study period with ages between 14 days- 5 years=45,334		
Cincinnati=13,732	Oakland=25,247	Norfolk=6,355

36%

Patients screened=16,145		
Cincinnati=7,402	Oakland=5,857	Norfolk=2885

15%

Patients enrolled=2,408		
Cincinnati=1,128	Oakland=991	Norfolk=289

76%

Patients tested=1,844		
Cincinnati=786	Oakland=813	Norfolk=245

The racial distribution of the population was different among the three hospitals: in Cincinnati 74% of the admitted patients were white, 22% were African American, and 4% belonged to other ethnic groups, in Norfolk 48% of the patients were white, 47% were African-American, and 5% had different ethnicities. In Oakland a most broad racial diversity was present: 15% were white, 45% African-American, 6% Asian/Pacific Islander, and 33% were grouped under other races.
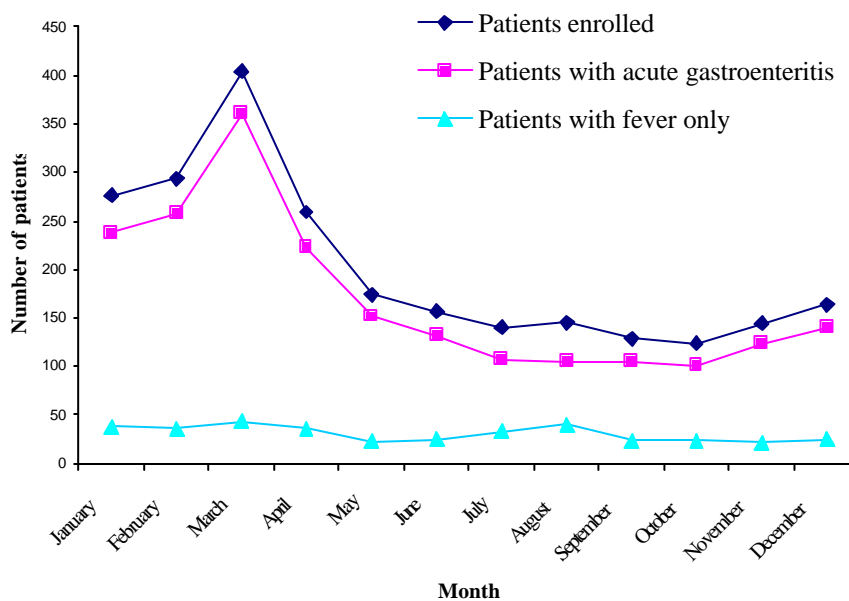
b) Enrolled patients

Annual distribution of enrollment showed a peak in February-March. This peak was caused by an increased number of patients with acute gastroenteritis during these moths while the number of patients with fever only enrolled during the study period did not show any seasonality (Figure 14).

The ethnic distribution of the enrolled patients was as follows: white 48%, African/American 28%, Hispanic 13%, Native American 0.1%, Asian/Pacific Islander 5%, and other races 6%. Ethnicity of enrolled patients was not different from ethnicity of the not enrolled patients at each site (P=.43).

Figure 14: Seasonal distribution of enrollment

Fifty-five percent of the enrolled patients were males. The age distribution of the enrolled patients was notably asymmetric with 75% of the patients less than 2 year-old (Figure 15). Among the enrolled patients, those with fever only were significantly younger than those with acute gastroenteritis (P<.001).

Figure 15: Age distribution
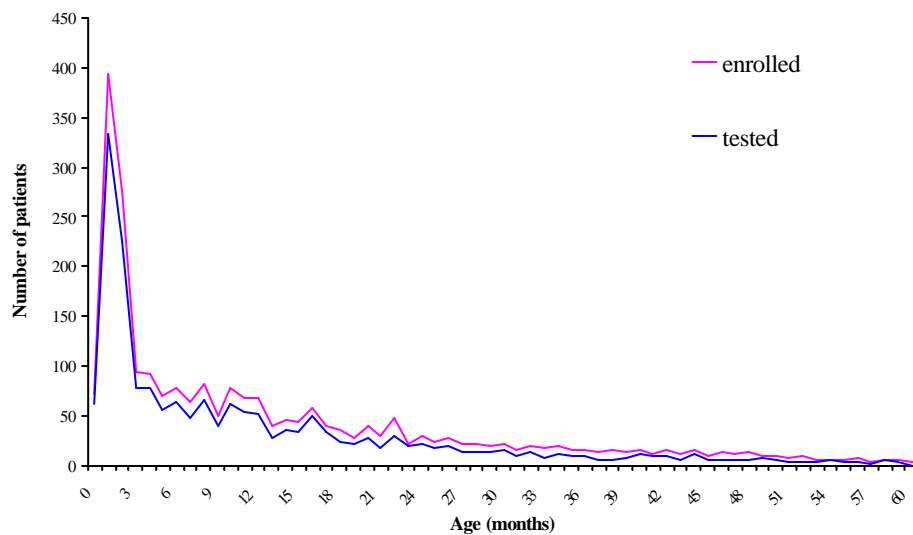
Twenty-eight percent of the enrolled patients had a chronic disease, 26% had history of prematurity, and 28% reported previous hospitalizations (other than birth, and not related to the current illness). Regarding clinical presentation, 2045 patients (85%) were admitted to the hospital with acute gastroenteritis, and 363 (15%) presented with fever only. According to the nurses' assessment, in 41.3% of the enrolled patients acute gastroenteritis was the primary cause for the admission, in 23.1%, gastroenteritis was present but was not the primary cause, in 15.4%, gastroenteritis was incidental, and in 20.2%, gastroenteritis was not a feature.

History of breast-feeding was reported in 55% of the enrolled patients with a median duration of 8 weeks and 75% of these children were breast-fed exclusively during a median period of 6 weeks. Four hundred and one patients (17%) were breast-fed at the time of admission (ages from 14 days to 44 months, median 54 days). The proportion of patients with acute gastroenteritis among the breast-fed

children at the time of admission was lower than the proportion among the non-breast-fed children (14% versus 70%, P<.001).

c) Patients tested

Stools were available for testing for calicivirus in 1844 patients (76% of all enrolled patients). Norfolk and Oakland had a higher percentage of patients with stools available for calicivirus testing than Cincinnati (P< .001). Gender, chronic disease, history of breast-feeding, breast-feeding at the time of admission, and presence of fever and/or diarrhea was similar in patients with a stool available for testing and those without stool (P>.05). Patients with a stool available for testing were significantly older and had a lower incidence of vomiting than patients with no stool available, P<.001. Out of the total stools available for testing, 1541 (84%) were from patients with acute gastroenteritis and 302 (16%) were from patients with fever only.

4.1.2. RT-PCR RESULTS

One thousand eight hundred and forty-five samples were tested for calicivirus by RT-PCR. Ninety-four samples were positive using primer pair 289/290 (5.6%).

Two hundred samples negative by 289/290 were selected to be tested with other primers: 289a/290a and 289hi/290hijk. The results with these two primer sets are summarized in Table 5.

		289hi/290hijk			
		Positive	Questionable	Negative	Total
289a/290a	Positive	6	0	2	8
	Questionable	1	0	0	1
	Negative	3	3	185	191
	Total	10	3	187	200

Table 5: RT-PCR results using p289a/290a and 289hi/290hijk in 200 samples negative by 289/290

Nine out of ten samples positives by 289hi/290hijk could be confirmed by sequencing while only six out of eight samples positive by 289a/290a could be confirmed (Figure 16). Because the sensitivity and specificity of primer set 289hi/290hijk looked to be better in our study population than for 289a/290a, all the samples negative by 289/290 were re-tested using 289hi/290hijk.

Figure 16: Sequencing confirmation of the samples positive or questionable for any of the two primer sets.
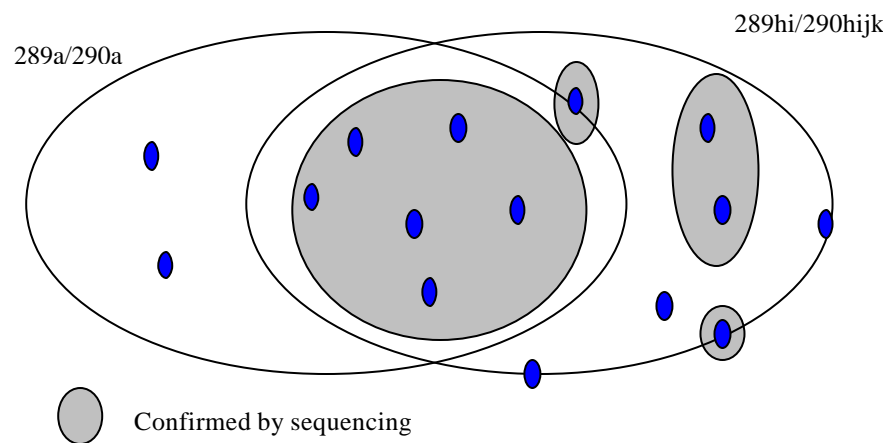
Out of 1790 samples previously negative by 289/290, 73 were positive when tested by primer set 289hi/290hijk. Sixty-eight samples positive by 289/290 were re-tested with 289hi/290hijk, only 38 samples were positive using the second primer. Overall, 167 samples (9%) yielded a positive result in the RT-PCR using any of the two detection primers (Figure 17).
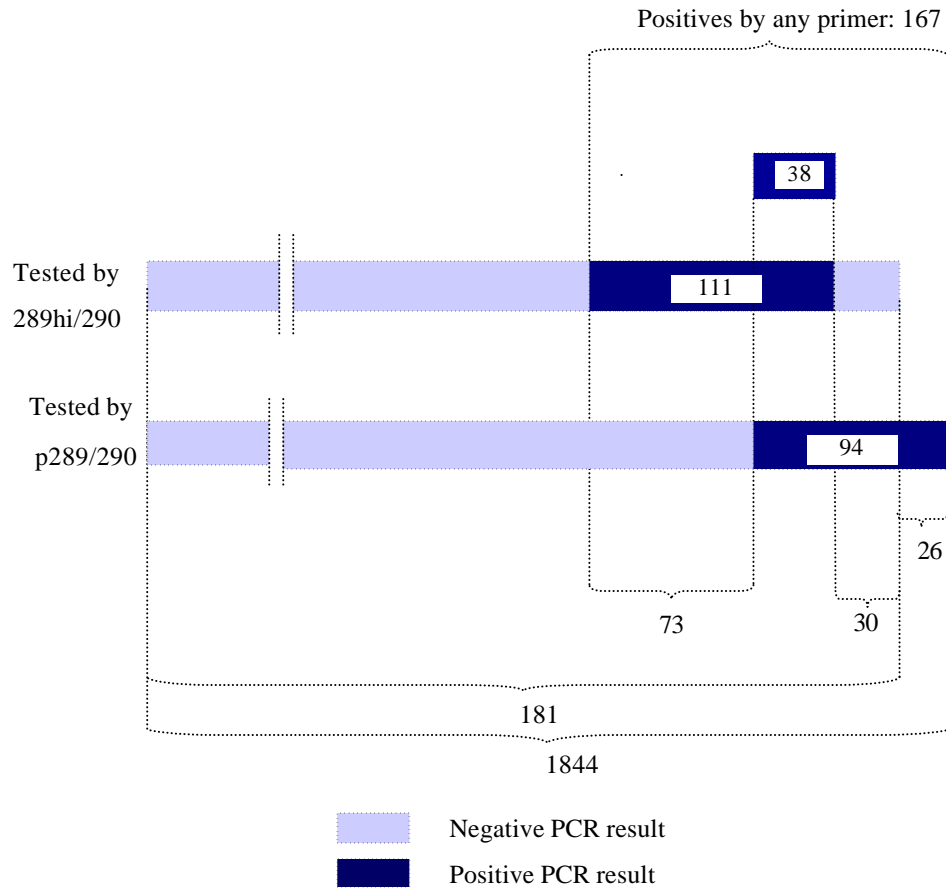
Figure 17: RT-PCR results in the complete study population using primer set 289hi/290hijk and 289/290.

Confirmation of PCR results

Confirmation by sequencing the PCR amplicon was attempted on all the positives by RT-PCR (167): 116 amplicons obtained with primers 289/290 or 289hi/290hijk were confirmed to be calicivirus, 35 amplicons could not be ligated or cloned, and in 16 of the amplicons, the sequence obtained was not a calicivirus sequence (Figure 18).

```
                          1844 samples
                               |
                               v
                          +----------+
                          |  RT-PCR  |
                          +----------+
                               |
                 +-------------+-------------+
               ( + )                       ( - )
                 |                           |
               <94>                       <1750>
                 |                           |
          +------+------+                    v
        <68>          <26>          +------------------+
          |             |           |     RT-PCR       |
          v             |           |  289hi/290hijk   |
    +----------+        |           +------------------+
    |  RT-PCR  |        |                    |
    +----------+        |          +---------+---------+
         |            ( + )      ( + )              ( - )
    +----+----+         |          |                  |
  ( + )    ( - )      <73>         |               <1677>
    |         |          \         |
  <38>      <30>          \        |
                           v       v
                      +------------------+
                      |       167        |
                      +------------------+
              Yes=116 ----+
                          |   +------------------+
                          |   | Confirmation by  |
   +-----------+  No=51 --+   |   sequencing     |
   | Seminested| <-----       +------------------+
   |    PCR    |
   +-----------+
```
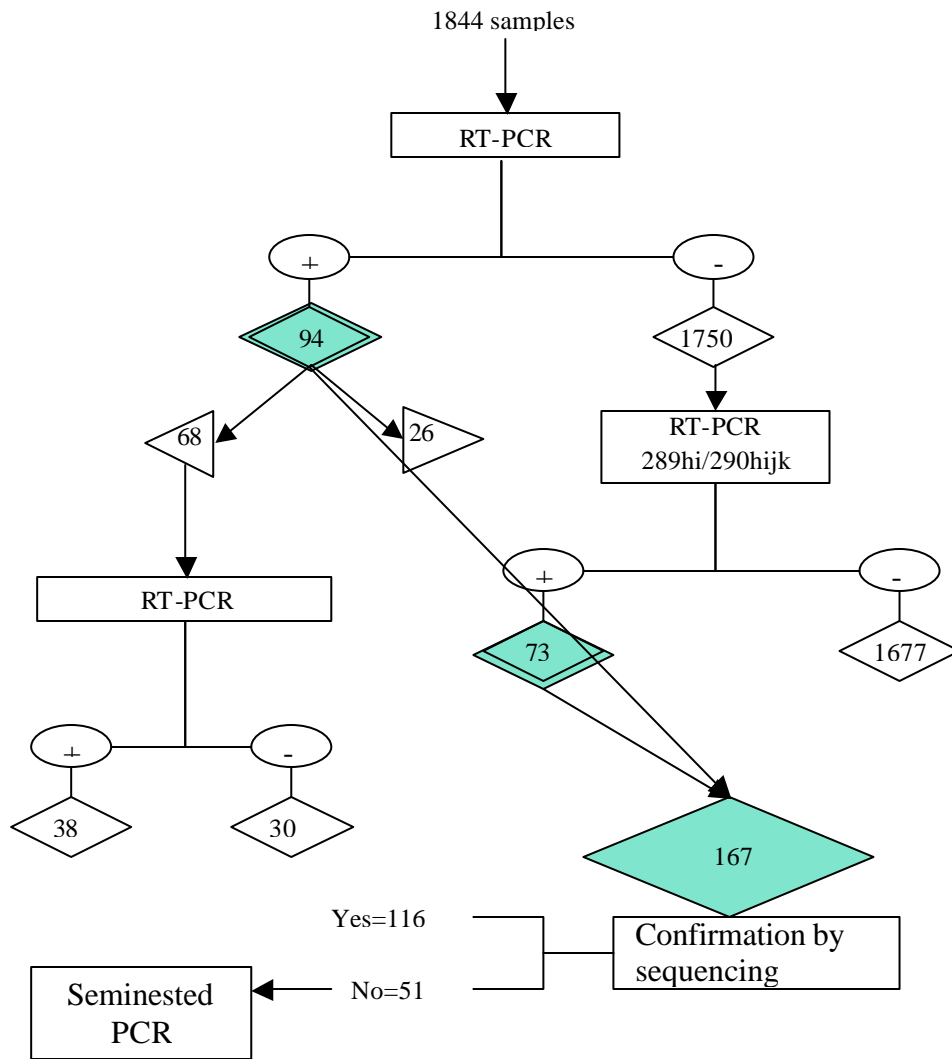
Figure 18: Confirmed original RT-PCR results

A seminested-PCR was performed on all the amplicons that could not be confirmed as calicivirus (51 samples) with the following results: 31 out of the 35 samples that could not be ligated/cloned were positive and 10 out of the 16 samples that showed a non-calicivirus sequence when the product obtained with primer 289/290 or 289hi/290hijk was sequenced were also positive. Overall 41 (82%) amplicons gave a positive result in the seminested-PCR. Sequence-

confirmation of the positive results obtained by the seminested-PCR was also attempted (41 samples): 39 amplicons were confirmed to be calicivirus, in one case the amplicon could not be ligated/cloned, and in 1 case, even when the positive result in the seminested-PCR supported the existence of calicivirus, the sequence obtained was not a calicivirus sequence (Figure 19).

Figure 19: Results and sequence confirmation of the seminested PCR on the amplicons that could not be confirmed in the original RT-PCR product
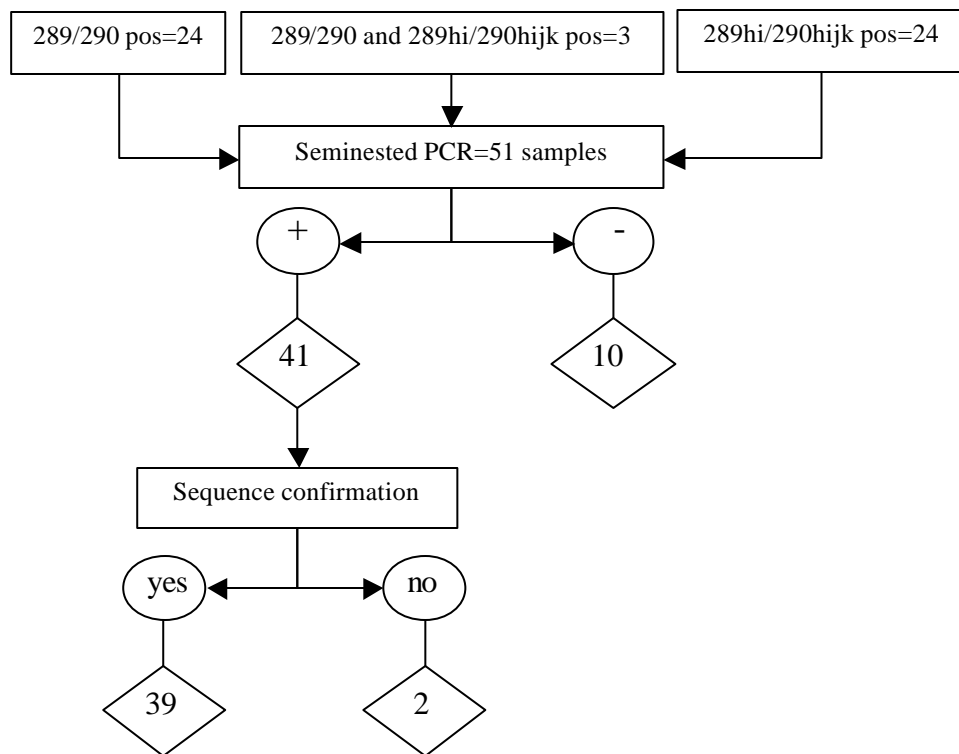
Overall, 155 out of 167 positive results by RT-PCR with any of the two detection primers were confirmed by sequencing (93%). Two of the positives by primer pair 289/290, 9 of the p289hi/290hijk-positives and 1 sample positive by both primers could not be confirmed. Two amplicons could not be ligated/cloned, and in 10 samples the sequence obtained was not a CV sequence. Ten out of the 12

samples that could not be confirmed as calicivirus were negative when tested by seminested-PCR.

The 10 non-calicivirus sequences were mainly ribosomal RNA from bacteria belonging to the CFB group (*Bacteroides-Cytophaga-Flexibacter*) and to the *Enterobacteriaceae family*. One sequence turned out to be human astrovirus.
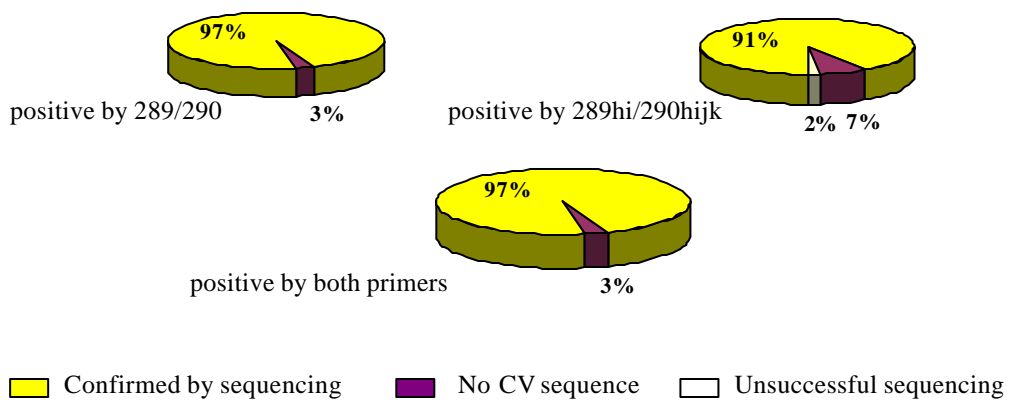
Out of 155 calicivirus positives, 130 were Norwalk-like viruses and 25 Sapporo-like viruses, resulting in a ratio of NLV: SLV 5:1.

Comparison of primers for detection of human calicivirus

The global detection rate was 5.6% for primer pair 289/290 and 6.3% (111 out of 1818 tested) for primer set 289hi/290hijk: 54 samples were positive only by 289/290 (26 samples tested by both primers and 28 samples tested only by 289/290), 73 samples were positive only by 289hi/290hijk, and 38 were positive by both primers. Using the sequencing results as a gold standard, the positive predictive value for primer pair 289/290 was 96.9%, for primer set 289hi/290hijk 90.9%, and for the combination of both, 97.4% (Figure 20).

Primer pair 289/290 showed a better sensitivity for SLV than 289hi/290hijk, while it was the other way around for NLV: RT-PCR using primer pair 289/290 detected 21 (84%) of the SLV-positive samples. Out of the 19 SLV that were tested by primer set 289hi/290hijk, 13 (68%) produced a positive result. RT-PCR by 289hi/290hijk detected 4 SLV that had not been detected by 289/290 but missed

Figure 20: Positive predictive value for each primer

positive by 289/290   97%   3%

positive by 289hi/290hijk   91%   2% 7%

positive by both primers   97%   3%

☐ Confirmed by sequencing   ☐ No CV sequence   ☐ Unsuccessful sequencing

6 that had been detected by the first primer. RT-PCR with 289/290 detected 71 NLV (55% of the confirmed NLV). Of the 106 NLV that were tested by p289hi/290hijk, 88 (83%) yielded a positive result (Figure 21).

Figure 21: Sensitivity of each primer for detection of Norwalk-like virus (NLV) and Sapporo-like virus (SLV)
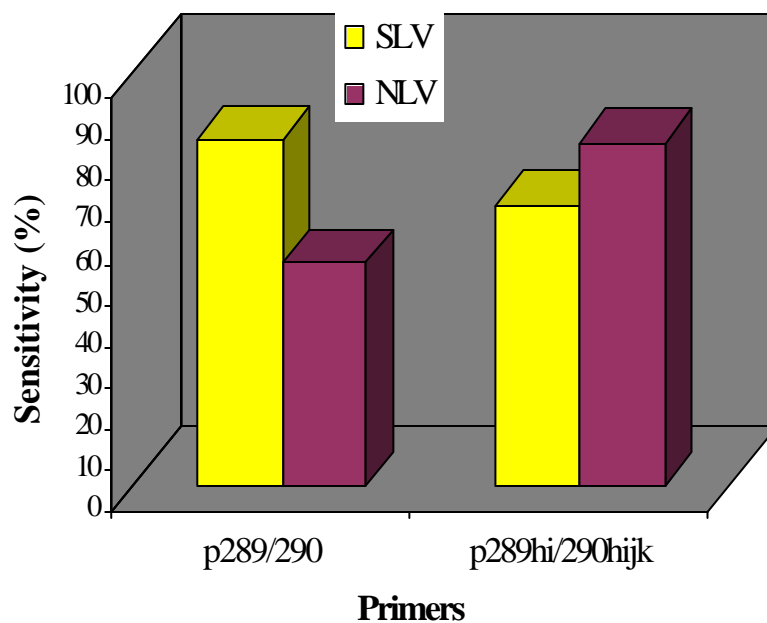
4.1.3. EPIDEMIOLOGY OF CALICIVIRUS INFECTION

Calicivirus was detected in 155 (130 NLV and 25 SLV) of 1844 samples tested (8.4%). There was no significant difference in the detection rate among the three sites (9.4% in Cincinnati, 7.9% in Oakland, and 6.9% in Norfolk, P=0.4).

The median age of patients infected by calicivirus was 262 days (range 14 to 1426 days). Patients infected only by calicivirus were younger than those infected exclusively by rotavirus (P<. 001). When considering patients with fever only there

was no difference between the two viruses regarding the age (Table 6). Age distribution was similar for patients infected by SLV and NLV: median age for SLV-infected children was 259 days and median age for NLV-infected children was 266 days (P=.46).

Table 6: Age distribution of patients with calicivirus and /or rotavirus infection

		N	median	Q25% -Q75%	
Tested					
	RV-positive	435	391	201-656	
	CV-positive	152	270	82-562	
	RV-positive only	417	390	200-649	p<0.001
	CV-positive only	134	228	64-526	
	RV/CV-positive	18	489	319-800	
	No virus detected	1275	135	44-455	
AGE					
	RV-positive	421	412	221-661	
	CV-positive	141	304	103-568	
	RV-positive only	403	405	220-661	p<0.001
	CV-positive only	123	257	82-531	
	RV/CV-positive	18	497	319-800	
	No virus detected	973	195	52-517	
Fever only					
	RV-positive	14	29	21-46	
	CV-positive	11	50	34-87	
	RV-positive only	14	29	21-46	p=0.1
	CV-positive only	11	50	34-87	
	RV/CV-positive	0			
	No virus detected	302	51	31-546	

Males were at no greater risk of calicivirus infection than females [RR=.99, 95%CI (0.96, 1.0) P=.3321]. None of the ethnic groups showed an increased risk for calicivirus infection in our study being the race distribution in the calicivirus infected patients and in non-infected patients similar (P=.21).

There was no difference in the incidence of calicivirus infection in patients with and without an underlying disease: calicivirus detection rate in patients with underlying disease was 9.8% versus 7.9% in previously healthy children (P= 0.16).

Sixteen percent of the patients infected by calicivirus were breast-fed at the time of admission versus 18% in the non-infected children. The incidence of calicivirus infection in patients who were breast-fed at the time of admission was 7.4% versus 8.6% in the ones who were not breast-fed at the time of admission (P=.43)

Out of the 155 positive samples, 10 (6.4%) were patients with fever only and 145 (93.5%) patients with acute gastroenteritis. The detection rate of calicivirus among patients with fever only was 3.4% and among those with acute gastroenteritis was 9.5%, P<.001. Two out of 25 patients infected with SLV (8%) and 9 out of 130 infected by NLV (7%) belonged to the group with fever only (P=0.69).

The seasonal distribution of calicivirus cases showed two peaks: one in early winter and another during spring time. This seasonal pattern was produced by NLV, while SLV infections did not show any seasonality in our study (Figure 22).

Figure 22: Seasonal distribution of calicivirus cases
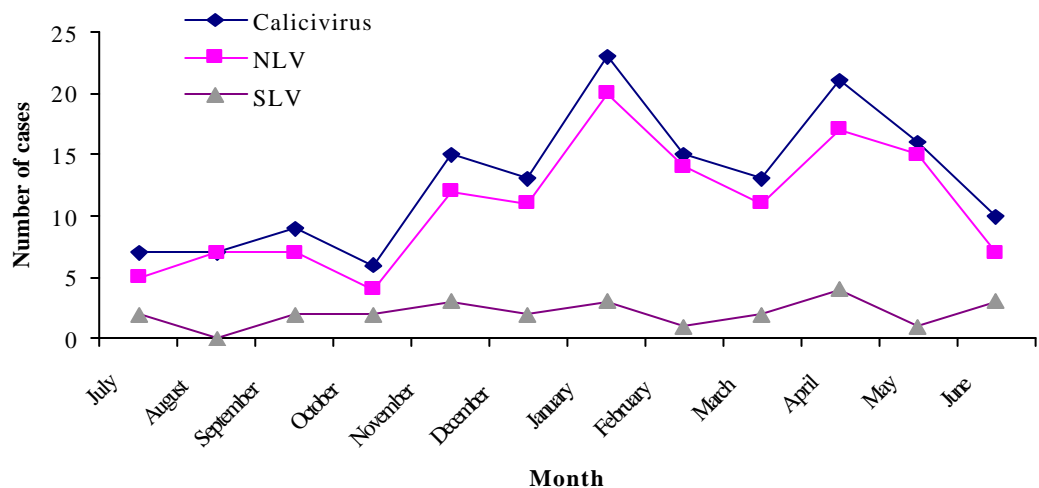
4.1.4. CLINICAL CHARACTERISTICS OF CALICIVIRUS INFECTION

Vomiting was present in 120 patients out of the 155 patients infected by calicivirus (77%). The median duration of vomiting was 1 day, with 10% of the

patients admitted to the hospital the same day of the onset of vomiting. The number of vomiting episodes per day ranged from 1 to 35 with a median of 6.

Diarrhea was a clinical symptom in 103 patients (66% of the calicivirus infected patients): in 97, diarrhea was present at the time of admission and in 6 patients diarrhea began one day after the admission. The median duration of diarrhea was 2 days, with a range from 1 to 50 stools per day (median number of stools per day= 6).

Fever was present in 83 children (54%) with a median duration of 1 day. In 25% of the patients, the day of onset of fever was the same day of admission to the hospital. The percentage of patients with diarrhea, vomiting, and fever was similar in patients infected with NLV and SLV.

The incidence of the different symptoms among the patients infected with calicivirus only, rotavirus only, co-infections, and those with no viral agent detected is summarized in Table 7.
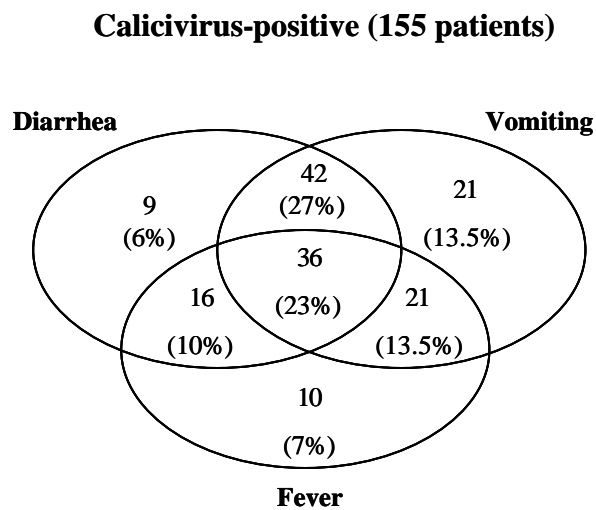
Table 7: Symptoms in patients infected by calicivirus, rotavirus, and co-infections in the study population (tested) and in patients tested with acute gastroenteritis (AGE)

		N	Fever	Vomiting	Diarrhea
Tested					
	Total	1844	1240 (67%)	1226 (66%)	1249 (68%)
	CV-pos	152	80 (53%)	119 (79%)	101 (67%)
	RV-pos	435	399 (92%)	390 (90%)	399 (91%)
	CV-pos pure	134	68 (52%)	100 (76%)	84 (64%)
	RV-pos pure	417	298 (72%)	371 (89%)	382 (92%)
	CV/RV-pos	18	12 (63%)	19 (100%)	17 (89%)
	No virus	1277	862 (67%)	736 (58%)	766 (60%)
AGE					
	Total	1517	913 (60%)	1226 (81%)	1225 (81%)
	CV-pos	141	70 (50%)	119 (84%)	100 (71%)
	RV-pos	421	296 (70%)	390 (93%)	397 (94%)
	CV-pos pure	123	58 (47%)	100 (82%)	83 (68%)
	RV-pos pure	403	284 (71%)	371 (92%)	380 (94%)
	CV/RV-pos	18	12 (63%)	19 (100%)	17 (89%)
	No virus	973	559 (57%)	736 (76%)	745 (46%)

When considering the group of children younger than 3 months of age infected with calicivirus, the most frequent symptom was fever (76%) while vomiting and diarrhea were present in only 56% and 51% of the patients, respectively. In patients older than 3 months of age, the most common symptom among the calicivirus infected patients was vomiting (86%), followed by diarrhea (67%) and fever in only 45% of the cases.

The most frequent clinical presentation at admission of the calicivirus infected children was vomiting with diarrhea (26%), followed by diarrhea with vomiting and fever (23%). In patients younger than 3 months, the most common clinical presentation was vomiting with fever (22%) followed by fever only (18%) (Figure 23). The least common presentation in children less than 3 months was vomiting alone (4%). In the older group - more than 3 month of age -, diarrhea with vomiting was the most frequent presentation at admission (31%), followed by combination of the three symptoms in 25% of the patients, and by vomiting alone in 17%. Fever only was the clinical presentation of 3% of the infected children older than 3 months (Figure 24).

Figure 23: Clinical presentation of calicivirus infection.

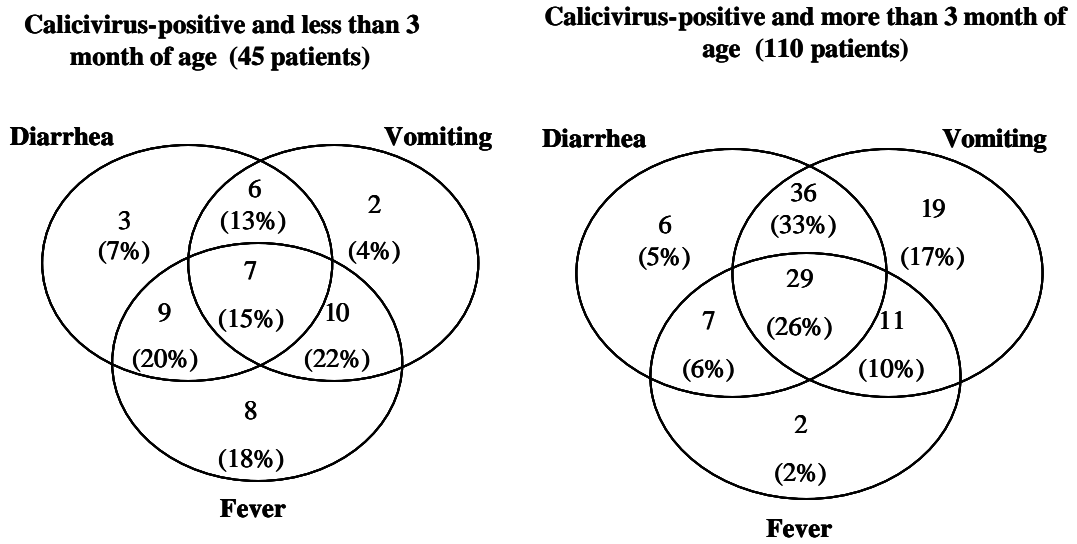**Calicivirus-positive (155 patients)**

Figure 24: Clinical symptoms by age group in patients infected by calicivirus.

Among the patients with all three symptoms present at the time of admission (36 patients), vomiting alone or in combination with other symptoms was the first clinical manifestation (83%), 27% presented with diarrhea and vomiting as the earliest symptoms with later onset of fever, and in 22% of the patients the three symptoms occurred at the same time. Only one patient had fever alone as a first symptom. In 50% of the patients with diarrhea and vomiting, both symptoms began at the same time, vomiting was first in 31% of the cases, and fever was the initial symptom in 17% (for one patient there was no information available). Among the patients with fever and vomiting, the chronology of the onset of symptoms was as follows: in 67% both symptoms began at the same time, in 19% vomiting was previous to the onset of fever, and only in 10% fever alone was the initial symptom. In patients with fever and diarrhea, 44% had diarrhea alone as initial symptom, in 37% both symptoms began at the same time, and in 19% fever alone was the original symptom.

The severity score (modified Vesikari score system) of patients infected with calicivirus and acute gastroenteritis ranged from 5 to 15 with a median of 9 points. Younger children (less than 3 month-old) had lower scores than older children:

median 8 versus 9 points (P<.001). The severity of gastroenteritis caused by NLV and SLV was similar (P=0.43).

Forty-two patients with calicivirus infection were admitted in the hospital with the diagnosis of dehydration: in one dehydration was associated with hypoglycemia, in another with hypocalcemia, and in 2 with metabolic acidosis. In two thirds of the patients with dehydration, a specific cause related with acute gastroenteritis, was coded in the admission diagnosis.

In 38 patients the admission diagnosis was rule out sepsis and/or fever with no other symptoms or diagnosis coded. In 6 patients urinary infection was suspected, in 4 pneumonia, in 1 meningitis, and in 4 otitis media. Fifteen patients were admitted with diagnosis related to respiratory symptoms without mention of fever or other symptoms compatible with acute gastroenteritis (bronchiolitis, asthma, respiratory distress, upper airway obstruction). In six of the calicivirus infected patients the reason for admission was seizures. The rest of patients had as an admission diagnosis vomiting and/or diarrhea and/or abdominal pain with no other symptoms/diseases associated.

Length of stay in the hospital for the children infected with calicivirus ranged from 1 to 33 days (median=2) with no difference between the three sites (P=.76) or between patients with gastroenteritis and patients with fever only (P=.15). Severity was not correlated with length of stay in these patients. There was no difference between NLV- and SLV-infected children regarding this factor (P=.75).

Eighty-nine patients with calicivirus infection were discharged with a non-specific diagnosis of viral disease, viral enteritis, non-infectious gastroenteritis, infectious gastroenteritis, vomiting, fever, or diarrhea. Thirty-one patients had a discharge diagnosis related with upper/lower respiratory tract infection, 6 patients were diagnosed of urinary tract infection, 5 patients of rotavirus infection, 2 of aseptic meningitis, and 2 of bacteremia. Hypovolemia was one of the first three discharge diagnoses in 65 of the 155 calicivirus infected children, 10 of them had an associated disorder in the electrolyte and/or acid-base balance.

A 7 month-old patient with congenital heart disease (canal AV), admitted in the hospital with vomiting and fever die after 33 days in the hospital. The diagnosis given at death was congestive heart failure.

4.2. <u>GENETIC DIVERSITY OF CALICIVIRUS CAUSING INFECTION IN CHILDREN</u>

Strains from both human calicivirus genera, NLV and SLV, were detected in the study population. NLVs were more frequently found than SLVs: 81% of the isolates in Cincinnati, 86% in Oakland, and 88.2% in Norfolk belonged to the NLV genera. (Figure 45).

Figure 25: Distribution of NLV and SLV at the three sites
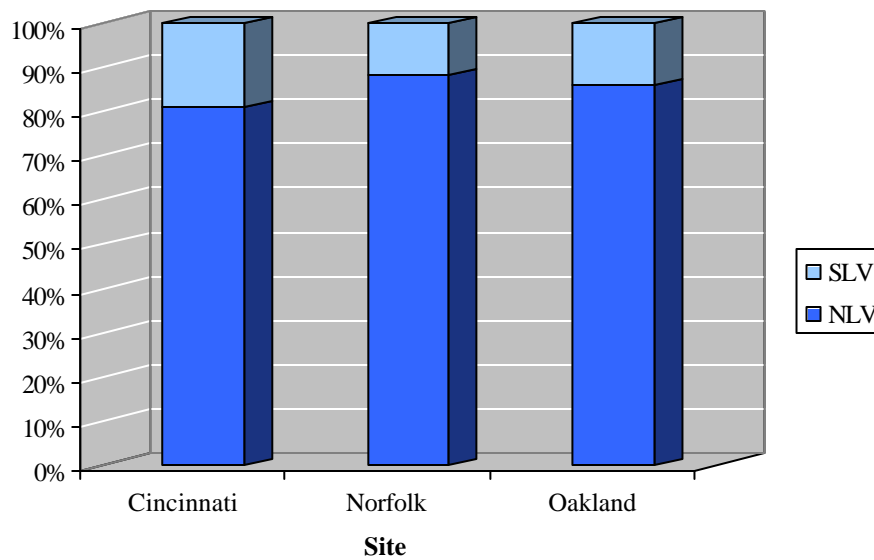
Within SLVs, three different genetic clusters or genotypes represented by London/92, Sapporo/82, and Houston/90 have been described.

Twelve of the SLV sequences (48%) fell in the London/92 cluster. This predominance of London-like strains occurred at all three sites with about 50% of the SLVs belonging to this cluster at each site. Five of the seven London-like strains isolated from Cincinnati were detected within an 8-month period (April-December 1998) and the other two were detected with a 15-day interval in September-October 1999. In Oakland, all four London-like strains detected were clustered in a 7-month interval (November 1998-June 1999) with no other strains

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of this genotype during the rest of the 2-year study period. Strains genetically very close (98% identical at nucleotide and amino acid level) were detected in Cincinnati and Oakland. Neither racial predisposition nor distinct symptomatology was apparent among the patients infected with these strains.

Six strains were grouped in the Sapporo/82 cluster: two of them 100% identical and isolated in Cincinnati from patients admitted in the hospital during the same week. No more SLVs belonging to this cluster were detected in Cincinnati until 18 month later. The other three strains belonging to this cluster were detected in Oakland without a clear temporal relation.

Three strains Houston/90-like were detected during the two-year study period, one in Oakland and 2 in Cincinnati with no temporal relation.

Three SLV strains, quite similar between them but not identical, did not fall in any of the three described genotypes. The samples were from different periods of time and geographic location: two samples from Cincinnati with collection date July 98 and February 99, and one from Norfolk collected in March 99. These three strains were 72% and 73% identical to Houston/90 and London/92, respectively. The identity between the Houston/90 and London/92 in this region is 71% being the new cluster equidistant from the reference strains of the two genotypes (Figures 26 and 27).

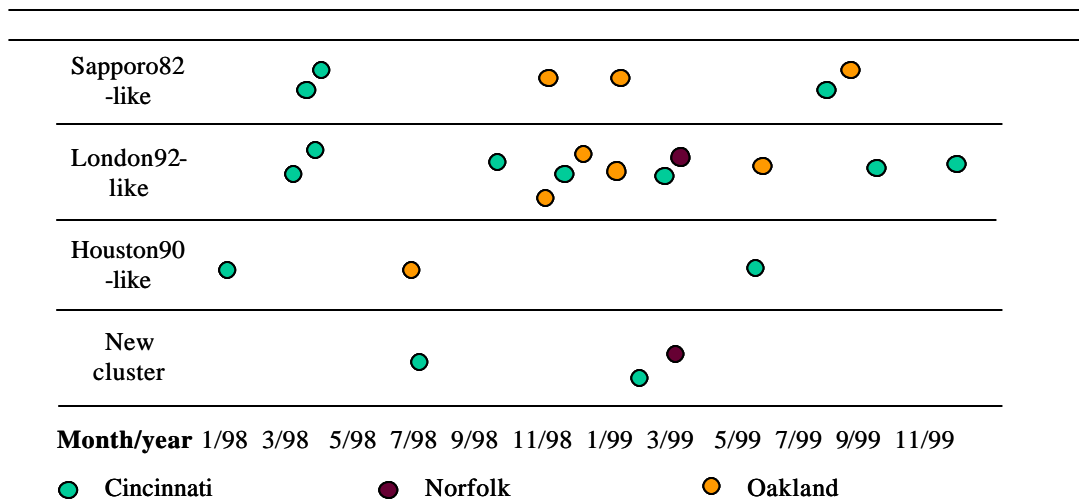**Figure 26: Time and site distribution of Sapporo-like strains**

Figure 27: Phylogenetic tree of Sapporo-like strains (Maximum likelihood algorithm with global rearrangement and bootstrapping 10 times). Blue circles represent already described clusters: Sapporo82-like, Houston90-like, and London92-like. The cluster highlighted in orange represents a new cluster, equidistant from other clusters described up to date.
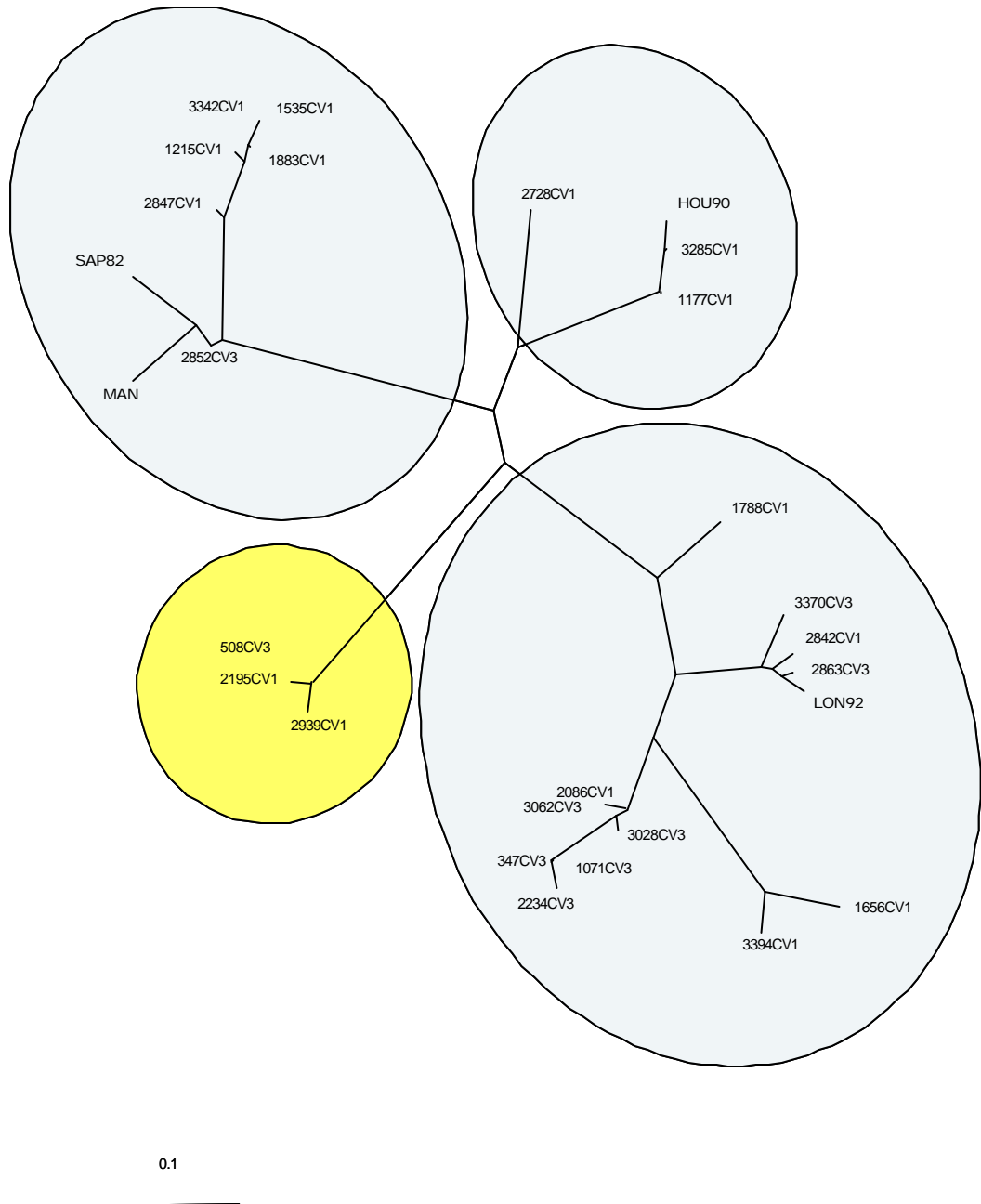
Regarding NLV, strains belonging to the two described genotypes: genotype I represented by NV, Southampton virus and Desert Shield virus, and genotype II represented by Lordsdale, MX, MOH, and Hawaii virus were present, being genotype II more frequently detected (Figure 28).

Seven strains fell in genotype I, six from Cincinnati and one from Oakland. No strains belonging to genotype I were detected in Norfolk during the 2-year study period. The clinical symptomatology in patients infected by strains belonged to this genotype was similar to those infected by strains belonging to genotype II and affected patients had ages from 20 days to 3 years old. No racial predisposition was apparent for infection by viruses belonging to this genotype.

One hundred and sixteen strains belonged to genotype II, mostly grouped in the Lodsdale-like cluster. Strains belonged to this genotype were detected in the three sites and during the two-year study period. Strains genetically very similar (95% homology at nucleotide level) were detected in different sites and in distant periods of time.

Seven strains did not fall in any of the two genotypes showing less than 65% identity at nucleotide level to any of the reference strains for each genotype. Among theses distinct strains, two different clusters were apparent: one cluster with a unique strain, isolated from a patient from Cincinnati admitted at the hospital in December 1997, and another cluster grouping six viruses, one from Norfolk with collection date May 1998 and five from Oakland with collection dates between November 1998 and November 1999. These strains neither could be grouped to any of the known clusters nor to the cluster represented by the animal Norwalk-like virus Jena virus. Four out of six patients presented a combination of diarrhea, vomiting, and fever. None of the patients was less than three months old. Two patients were white, two African-American, two Hispanics and one belonged to another ethnic group.

Figure 28: Phylogenetic tree obtained applying Maximum Likelihood to the NLV strains isolated in our study. Reference strains for genogroup I, DSV, NV, and Southampton, and for genogroup II, Lordsdale, MOH, HA, and MX have been included.
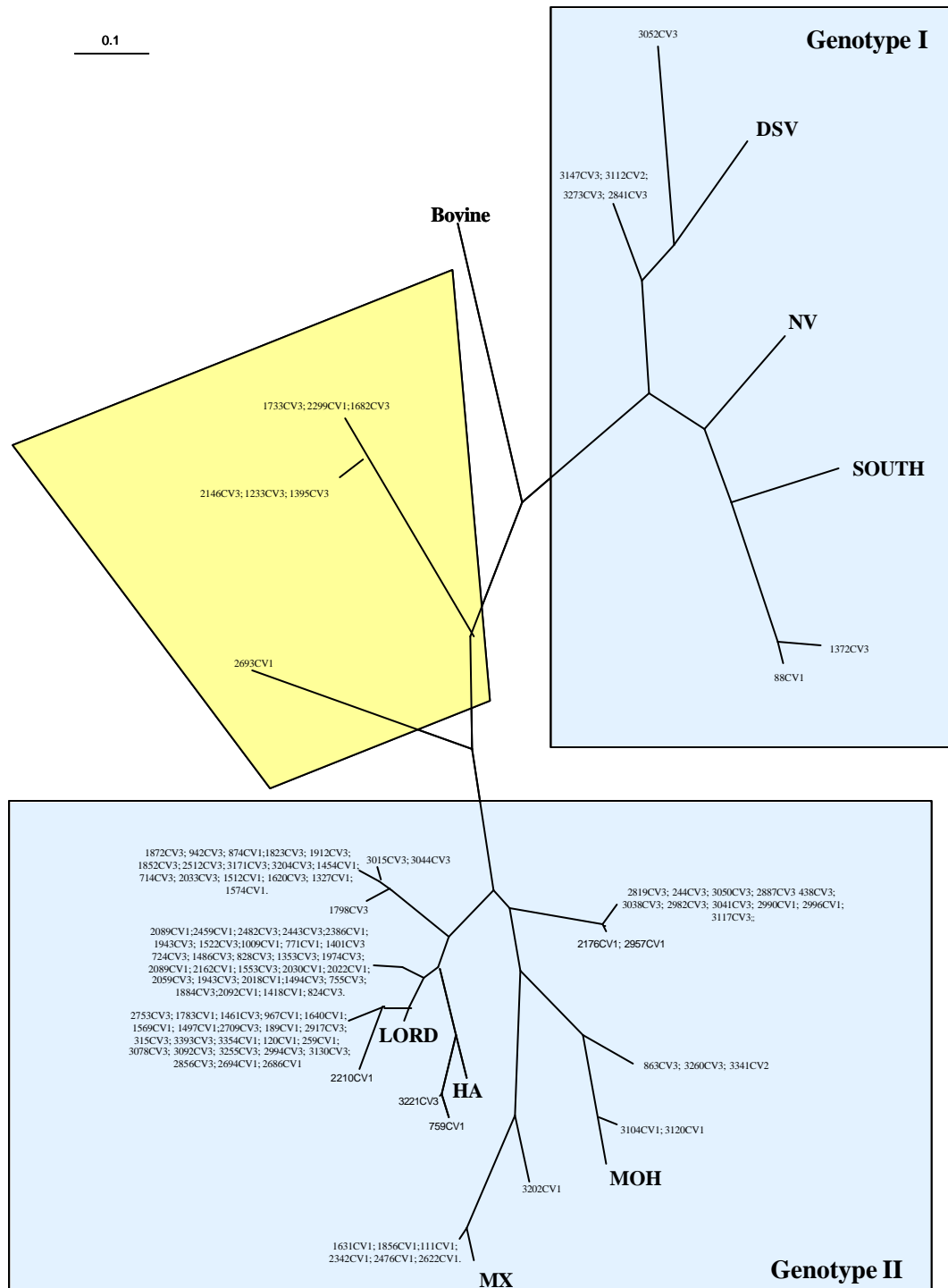
4.3. <u>MEDICAL COST ASSOCIATED WITH CALICIVIRUS INFECTION</u>

The different components contributing to the medical cost in each period are summarized in Table 8.

Table 8: Components contributing to the medical cost in the pre-, hospitalization, and post-hospitalization period

	Pre-hospitalization		Hospitalization	Post-hospitalization	
	No. of patients	No. of ocurrences	n=155	No. of patients	No. of ocurrences
Visits to a health care provider	82	120	~	100	135
Physician's office	55	63	~	83	104
ER	31	40	~	5	5
Clinic	4	5	~	17	18
Hospitalization	4	4	~	5	5
Other	2	8	~	1	3
Tests ordered	35	80	N/A	14	21
Blood tests	29	33	N/A	10	12
Stool tests	9	9	N/A	3	4
X-rays	16	19	N/A	2	2
Other	16	19	N/A	3	3
IV-fluids	22	22	N/A	6	6
Medications	50	66	N/A	41	55
Cost per patient ($)					
Median		39	3,325		39

4.3.1. Pre-hospitalization period

Eighty-two patients (53%) consulted at least once to a health care provider because of the current illness with an average of 1.4 visits per patient: 55 consulted in one occasion, 19 twice, 5 in three, and 3 patients had sought medical care more than three times prior to be admitted to the hospital. Of the 120 occurrences, 64 (54%) were visits to the physician's office, 37 were emergency room visits, and 5 patients consulted to a clinic. Four subjects had been admitted to a hospital due to their current illness prior to the present hospitalization. The percentage of patients with no visits to a health care provider prior to admission to the hospital was much higher in Oakland (55%) than in Cincinnati (45%) and Norfolk (29%) although the difference did not reach statistical significance (P=.14).

Physicians ordered intravenous rehydration in 22 patients and tests in 35 patients during the pre-hospitalization period: 31 blood tests, 18 X-rays, 9 stool tests, 11 urine analysis or urine culture, 3 lumbar punctures, and one Streptococcus antigen detection.

Forty-nine patients received medications, mostly antipyretics (32 patients). Antibiotics were prescribed to nine patients; four were children younger than 3 month-old. The antibiotics prescribed were: amoxicillin in 5 patients, azythromycin in 1 patient, one patient received ceftriaxone followed by ampicillin, another patient cefpodoxime followed by cefprozil, and one patient received a prescription for cefprozil in the first visit, for amoxicillin in the second visit, and for amoxicillin with clavulanate in the third visit.

Thirty-nine percent of the calicivirus infected patients did not incur in any medical expense prior to hospitalization. The range for the ones who did have expenses was from $4 to $13,420, median $78. Overall, the median cost in the pre-hospitalization period for a children infected by calicivirus was $39 (25%-75% percentile: $0-$114.5) with no difference among the three sites (P=0.05)

4.3.2. Hospitalization period

There was information available for 152 patients regarding the costs in this period. The median cost for hospitalization was $3,325, ranging from $699 to $116.088.

There was differences in the hospital charges among the three hospitals even after adjusting with a geographical factor: the median cost per patient in Cincinnati was $3,035, in Norfolk $3,228, and in Oakland $4,067. The difference was only statistically significant between Cincinnati and Oakland (P=.003).

The cost was strongly correlated with the length of stay in the hospital (R=.72, P<.001), and showed a weak negative correlation with the age of the patient (R=-0.30, P<.001). No significant correlation between severity and cost of hospitalization was found. Having an active chronic condition was associated with higher hospitalization costs than patients with no active chronic disease (P=.02).

4.3.3. Post-hospitalization period

Ninety-nine patients (65%) had at least one follow-up visit: 73 patients had one visit, 20 patients two visits, and 6 three visits. One hundred and three out of 131 encounters (79%) took place in the physician's office, 6 patients were evaluated in the emergency room, and 5 patients had to be readmitted to the hospital. In Norfolk, 94% of the patients had at least a follow up visit, in Oakland 67% and in Cincinnati 55% (P=.009). Twenty-one tests were ordered during these follow-up visits: 12 blood tests, 4 stool tests, 3 X-rays, and 1 urine analysis.

During the post-hospitalization period costs due to medications were reported for 41 patients, 15 of them received a prescription for an antibiotic. The antibiotics prescribed were ampicillin IV, ceftriaxone, co-trimoxazole (2 patients), claritromicyn, penicillin, amoxicillin (4 patients), cefzil, and in 4 patients the antibiotic was not specified.

There was no report of medical expenses during the post-hospitalization period for 31% of the calicivirus infected patients. Among the patients with costs in this period the median was $46 (range $6 to $4290) and $39 (range $0 to $4290) when considering all calicivirus infected patients. The median medical cost in this period was higher in Norfolk ($45), than in Cincinnati and Oakland ($39), mainly due to the high proportion of follow up visits occurred in Norfolk (P=.02).

4.3.4. <u>Total medical cost</u>

The median medical cost for calicivirus infected patients was $3,574 (range $820-$116,088). The medical cost in Oakland was higher than in Cincinnati (P=0.003). No statistical difference was detected between Cincinnati and Norfolk (P=.28).

Patients with an active chronic disease had higher medical costs than patients with no active underlying disease: median $4,352 versus $3,318 (P=.002) and the total medical cost showed a weakly negative correlation the age of the patient (R= -0.19, P=0.02).

The global medical cost (pre-hospitalization, hospitalization, and post-hospitalization period) for the 152 infected patients with information available during this 2-year study was $780,920. This cost does not take into account the non-medical component associated with any medical condition, neither the cost associated to the death of one of the patients infected by calicivirus.

5. <u>DISCUSSION</u>

Challenge of calicivirus detection

Calicivirus was the first viral agent proved to cause acute gastroenteritis in humans[20]. Despite this promising debut, the role of caliciviruses in sporadic cases of acute gastroenteritis is still unknown. The usually self-limited course of viral gastroenteritis with no specific treatment available for this illness and the difficulty in making the causative diagnosis has lead to a lack of information regarding the epidemiology of this viral illness. The evidence that calicivirus causes large outbreaks of acute gastroenteritis with the subsequent economical and social impact, has raised an interest in getting a better understanding of the epidemiology, clinical manifestations, and genetic diversity of this virus.

The first problem faced when assessing the incidence of calicivirus infection is the lack of a universal, reliable, and clinically available method of detection. The inability to grow human calicivirus in animal models or in cell cultures and the antigenic and genetic diversity of theses viruses has led to the development of a variety of diagnostic methods, none of them free of inconveniences[20,43,50,109,110,113,117,129]. Currently, RT-PCR is the method of choice in the majority of laboratories.

Even though RT-PCR is used broadly, no universal primer pair capable of detecting all the different calicivirus strains has been found. Over 30 primer pairs have been published, some able to detect both NLV and SLV strains, others specific for either NLV or SLV, and others targeting strains belonging to a specific genogroup. The fact that there is no primer or combination of primers accepted as a gold standard for universal detection of human caliciviruses is illustrated by the diversity of the primers used by different laboratories, and by some studies showing that at least three different primer pairs must be used in order to detect the high variety of caliciviruses causing illness in humans. At the same time, scientists recognize the need for confirmatory methods due to the fact that some of the primers have been designed based on the YGDD motif, which is highly conserved among double stranded and single stranded RNA viruses, including calicivirus, rotavirus and enterovirus[130].

The primer pair chosen for the screening for human caliciviruses was 289/290 designed in our laboratory by Jiang. This primer pair is able to detect strains

belonging to both genera, SLV and NLV[119]. The detection rate in our study population using this primer pair was 5.6%. A subset of samples stratified by site, age and date of admission was screened to see if additional positives could be detected using other primers. Two hundred samples, negative by 289/290, were tested using 289a/290a and 289hi/290hijk, with a supposed better sensitivity for calicivirus than the existing primer pair 289/290. The first problem faced was that none of these new primers could detect primate calicivirus, a virus that was used as a positive control in the RT-PCR with 289/290 (primate calicivirus is a cultivable animal calicivirus so unlimited supplies with a determined concentration are available). This fact was the first indication that maybe the new primers would not be able to detect some of the positives by 289/290.

Another difficulty faced with the new primers was the presence of nonspecific banding in many samples, just slightly different in size from the expected product, and the presence of smears when the PCR amplicons were visualized in the agarose gels, making the results difficult to interpret.

A detection rate of 4 and 5% by 289a/290a and 289hi/290hijk respectively among the negative samples by 289/290, almost doubled the number of positives, which was quite striking. The presence of samples negative by primer set 289hi/290hijk but positive when tested by 289a/290a and vice versa indicated that the specificity of this primers was not optimal or that the spectrum a calicivirus detected by each one was different.

During confirmation of the RT-PCR results, the presence of nonspecific bands very close in size to the expected amplicon, made the sequencing process challenging. Nine out of 12 positives by any of the two primer pairs could be confirmed: 75% of the positives by 289a/290a and 90% of the positives by 289hi/290hijk.

One of the positives by 289a/290a, when confirmation was attempted, showed a poliovirus sequence with primer 289a present at both ends and just 2 nucleotides longer than the expected size for NLV. In the USA, where vaccination with polio live attenuated virus has been recently stopped, this nonspecific amplification would not be currently a problem, not so in other countries where live attenuated

polio vaccine is still in use leading to the excretion of polio virus in the stool during a quite prolonged period of time.

Overall, primer set 289hi/290hijk showed a better sensitivity and specificity in this subset of samples when compared to 289a/290a: none of the samples positive only by 289a/290a could be confirmed as calicivirus, while two of the three samples testing positive with 289hi/290hijk could be confirmed as true positive.

A detection rate of 4.2% positive samples by RT-PCR using 289hi/290hijk among the samples negative by 289/290 in the complete study population supposed a 60% increase in the detection rate of caliciviruses. This confirmed the suspicion that even 289/290, a primer pair able to detect strains belonging to all genogroups, it cannot detect the entire spectrum of caliciviruses causing infection in humans.

Although the detection rate using 289hi/290hijk was higher than the detection rate using 289/290 (6.3% versus 5.6%), the pool of primers is not a substitute but a complement for 289/290 since 289hi/290hijk failed to detect 44% of the positives by 289/290.

The need of a confirmation method for the RT-PCR results at this stage in the development of primers was strengthened by the fact the some of the amplicons generated in the RT-PCR were ribosomal RNA from bacteria belonging to the CFB group (*Bacteroides-Cytophaga-Flexibacter*) and *Enterobacteriaceae* family, commonly found as colonizers of the intestinal tract, and in one case the amplicon was astrovirus, a known cause of gastroenteritis.

Epidemiology of calicivirus infection in hospitalized children

Calicivirus is the leading cause of gastroenteritis outbreaks in the adult population[11,68,69]. The usual self-limited course of the illness and the association of these outbreaks mainly with adult populations has propitiated that most of the studies were conducted among adults in community settings. The role of calicivirus-associated gastroenteritis causing hospitalization in children has been addressed in a few studies but differences in the population characteristics (number of patients, age range, clinical presentation, country were the study was conducted, length and seasonality of the surveillance) and the diversity in the detection method (electron microscopy, RT-PCR, ELISA) lead to a substantial diversity in

the results, ranging from <1% in a study conducted by Nakata et al. in USA to 9% in a study conducted in Australia by Kirkwood[131][66].

The overall detection rate in our study population was 8.4%. The detection rate among patients with diarrhea was 8.2%, similar to the 7.6% detection rate obtained in a study conducted in China with a similar population using RT-PCR as the detection method [132]. The incidence of calicivirus infection in children with acute gastroenteritis in which rotavirus had been excluded was 11.5% in our study population, higher than the 9% reported by Kirkwood in hospitalized children with acute gastroenteritis negative for other pathogens[66].

Even the prevalence of calicivirus infection in our population was higher than reported previously, these results reflect a minimum prevalence and further testing with different primers would probably lead to the detection of more positive samples.

Rotavirus is a well-known cause of gastroenteritis in children while calicivirus has been associated more frequently with gastroenteritis in adults. The finding of 8% of calicivirus associated illness in our study and with an age significantly lower than the age of children infected by rotavirus supports our original hypothesis that the role of calicivirus infection in children is relevant and that this virus may represent a considerable burden in very young kids.

It is known that there is an individual predisposition to develop clinical illness when calicivirus infection occurs. This individual predisposition to develop clinical illness has been linked to the blood group antigens (ABO) in studies with volunteers (MK Estes, personal communication) and also to some degree of resistance in certain ethnic groups [133]. In our study, the ethnic distribution of the calicivirus infected children was similar to that of non-infected patients.

SLVs represented 16% of calicivirus detected in our population with no difference when comparing very young children (less than 3 month old) and older children. These results are in agreement with previous studies where SLV infection appeared to be less prevalent and also associated with a milder disease than NLV, and therefore, less likely to be found in hospitalized children[96].

The association of NLV with a syndrome known as a "winter vomiting disease" is a clear indication of the seasonality of this virus. Although sporadic

cases and outbreaks of gastroenteritis caused by NLVs and SLVs occur year round, the winter pattern has been reported repeatedly worldwide[82]. This seasonality, however, was not found in one study conducted by Nakata in an infant home in Japan during a 20-year period where 36 outbreaks were studied[46]. In our study, NLV was detected year round but with a clear drop during the summer (78% of the cases occurred between November and May). We could not demonstrate seasonality for SLV, probably due to the small number of cases detected.

Breast-feeding has proved to confer protection against severe disease caused by rotavirus. The role of breast-feeding in calicivirus infection is completely unknown and it will probably result in a complex question to elucidate in light of the current data available about the development of a protective immune response to this virus. In our study, the detection rate of calicivirus was similar in children who were breast-fed at the time of admission and in those who were not-breast-fed. The lack of a control group with no illness and presence of calicivirus in their stool, preclude drawing any conclusion about the protective role of breast-feeding in the development of symptomatic infection.

Clinical characteristics of calicivirus infection

Vomiting (alone or in combination with other symptoms) was the most frequent clinical manifestation in calicivirus infected patients (77%) with a broad range in intensity (from 1 to 35 episodes per day). Although several studies have reported that SLV is usually associated with mild diarrhea with little vomiting, 81% of our patients with SLV infections presented with vomiting. The fact that our study population just includes patients with severe illness could explain this discrepancy in the clinical presentation of SLV infection. Vomiting alone was the most unusual presentation among patients less than 3 months old, while in 17% of the patients older than 3 months infected by calicivirus, vomiting was the only clinical sign.

Fever was the most common symptom in children younger than 3 months of age infected by calicivirus (62%) and the only symptom in 10 patients (eight patients younger than 3 months and 2 older). Two of the 10 patients with fever only were discharged with a diagnosis of bacteremia and therefore calicivirus may

not have played a role as a cause of the illness. The detection of calicivirus in the stool of 8 patients with fever only and no identified clinical cause at the time of discharge could have different explanations: (1) these patients represent the population with asymptomatic infection described in previous studies and fever was caused by another pathogen or condition not identified during the hospitalization, (2) fever without gastroenteritis is a clinical picture of calicivirus infection not considered up to this date, (3) a combination of both.

Clinical presentation and severity in NLV- and SLV-infected patients was similar. When compared with rotavirus infection, calicivirus infection was less severe (median score for rotavirus was 11 and for calicivirus was 9), although this difference in score did not appear to be clinically significant to distinguish between the two because of the broad range and overlapping values among the two viruses: in rotavirus-infected patients the range of severity was 4-16 versus 5-15 in calicivirus infection. Co-infection with calicivirus did not increase the severity of rotavirus infection in our study population.

Calicivirus infection can be associated with severe vomiting (more than 10 episodes per day in 25% of the patients), diarrhea (more than 10 stools per day in 25% of the patients) and high fever (up to 40.5℃). None of the symptoms by itself was more severe in rotavirus than in calicivirus infection but a combination of the three symptoms was much more frequent in patients infected by rotavirus (58%) than in patients infected by calicivirus (23%).

Two patients with no gastrointestinal symptoms were discharged with a diagnosis of acute bronchiolitis with no specific cause identified. The association of SLV infection with respiratory symptoms has been previously reported, but always associated with gastrointestinal symptoms. The two patients with NLV infection may represent asymptomatic calicivirus infection associated with another viral infection causing the respiratory symptoms or may also represent a different clinical spectrum of calicivirus infection; this would be supported by the fact that 29 (20%) of the patients with gastrointestinal symptoms and calicivirus infection had a discharge diagnosis related to upper and/or lower respiratory tract infection with no etiology identified. Another interesting finding was the detection of calicivirus in the stool of two patients discharged with a diagnosis of viral

meningitis without an identifiable organism in the cerebrospinal fluid. Although there is no information about calicivirus causing extra-intestinal disease in humans, the fact that rotavirus, a virus with a clinical spectrum very similar to calicivirus, can involve the central nervous system [134,135] and the broad spectrum of illness caused by animal caliciviruses, including encephalitis, make us wonder about the role of calicivirus in the aseptic meningitis of these two patients.

Further studies are necessary to determine the incidence of asymptomatic calicivirus infection in children and to elucidate the role of this virus as a cause of fever (with no gastrointestinal symptoms) and other extra-intestinal manifestations.

Genetic diversity of calicivirus causing severe infection in children

The genetic diversity of calicivirus, even when they are analyzed in "conserved" regions, is one of the major obstacles to develop universal primers able to detect the broad spectrum of calicivirus causing disease in humans.

The study of the polymerase region, one of the conserved regions in the calicivirus genome, has advantages and inconveniences. The principal advantage of working with conserved regions is that it allows to develop primers able to detect the broad spectrum of calicivirus strains. A better knowledge of this conserved region will allow to develop universal primers based upon this region. The development of universal primers is actually one of the hot topics in the calicivirus study, and one of the most pressing demand of investigators and epidemiologists working in this field. The lack of a universal detection method lead to differences in detection rate, mostly due to different sensitivity of the primers than a real differences in the incidence that makes difficult to compare results published by different groups.

Even though the diversity in the polymerase region is lower than in the capside region it is now accepted that the information contained in this region is enough to allow the classification of the different strains at the genera and genotype level, follow outbreaks, and trace the origin of these outbreaks quite confidently.

Disadvantages of sequencing and analyzing the polymerase region are that being relatively conserved, the study of phylogenetic relationships among strains

requires quite complex algorithms, that the biologic meaning of nucleotide or aminoacid composition in this region in unclear (does not imply antigenic differences), and finally that the presence of recombination would generate disagreeing results when analyzing the polymerase and the capsid region.

This diversity of calicivirus was well represented in our study population. Strains belonging to Norwalk-like and Sapporo-like genera were present, and within them, a broad spectrum of strains could be studied. NLV were predominant (about 80% of the calicivirus belonged to this genera) and within NLV, genotype II was the most prevalent. This pattern has been previously reported by different authors[78-81]. Strains forming new clusters, in both, NLV and SLV genera, were detected. Phylogenetic distances with known clusters were comparable to distances that currently define, for many authors, different genotypes. To determine if these differences in the polymerase region have a biologic implication, sequencing of the capsid region, expression of the capsid in a baculovirus system, and study of antigenic properties of these new strains would be necessary. The fact that these new strains appeared in different sites with no temporal relation induces to think that this is not a unusual event, and probably there is a continuum in the genetic diversity of calicivirus, but due to the limitation in the actual detection methods, only determined strains are currently identified.

Medical cost of severe calicivirus infection

The median cost per episode of calicivirus infection in our study population was $3,574. The main component of this cost (more than 90%) was hospitalization. The cost of hospitalization showed a strong correlation with the length of stay but was independent of the age of the patient and of the severity of gastroenteritis. There were differences in the hospitalization cost, even after adjusting with a geographical factor, when the three sites were compared: the hospitalization costs were higher in Oakland and lower in Cincinnati. This difference may be explained by the existence, in the Cincinnati Children's Hospital, of a specific unit where patients with acute gastroenteritis are admitted. In that unit, established protocols

and discharge instructions for the nursing staff are in place, minimizing the need for physician intervention.

Although the cost of visits to a health care provider in the pre- and post-hospitalization period was not significant when compared to the hospitalization cost, the burden of 168 visits to a physician's office, 45 visits to the emergency room, and 23 visits to clinics in just 155 patients does not look so insignificant.

Twenty-one X-rays, 45 blood tests, and 13 stool tests were ordered during the pre- and post-hospitalization period for the 155 patients infected by calicivirus.

Medication was another component we looked at. The most common medication was antipyretics - medication with a low cost and side effects- but 9 patients in the pre-hospitalization period and 15 in the post-hospitalization period received antibiotics. Just 2 out of 9 patients treated with antibiotics during the pre-hospitalization period were discharged from the hospital with a diagnosis compatible with bacterial infection. In 4 of the patients that received antibiotics during the post-hospitalization period there was no diagnosis discharge indicating a possible bacterial infection either. Although these numbers are not very impressive, the use of antibiotics in viral infections generates a cost, not only monetarily, by increasing the bacterial resistance to antibiotics with the consequences that this implies.

6. <u>CONCLUSIONS</u>

1. Detection of human caliciviruses in clinical samples is still a challenge due to the lack of universal primers able to detect the broad diversity of viruses affecting humans with an acceptable sensitivity and specificity. The real incidence of calicivirus infection is probably underestimated due to the use of non-optimal detection methods.

2. Calicivirus infection could be proved in 8.4% of hospitalized children 14 days to 5 years old admitted because diarrhea, vomiting and/or fever. Excluding the patients with rotavirus infection, calicivirus was detected in 11.5% of the patients. Neither gender nor ethnicity appear to be a predisposing factor for calicivirus infection.

3. NLVs are more prevalent than SLVs in hospitalized children: 84% versus 16%.

4. Cases were detected all year round but with predominance during winter-spring. Among calicivirus, NLVs showed this seasonal pattern while SLVs did not.

5. Calicivirus infection can be associated with severe vomiting (more than 10 episodes per day in 25% of the patients), diarrhea (more than 10 stools per day in 25% of the patients) and high fever (up to 40.5ºC). Vomiting (alone or in combination with other symptoms) is the most frequent clinical manifestation in calicivirus infected patients.

6. Detection of calicivirus in patients with fever only or clinical manifestations different to those of acute gastroenteritis, suggests that the clinical presentation of calicivirus infection can be broader than previously reported.

7. Caliciviruses are genetically diverse and this diversity is present among strains causing severe disease in children. Strains genetically similar are detected in distant places and with broad time-intervals. Strains genetically diverse are present in a geographic location during the same time-period.

8. No differences regarding clinical presentation and demographic characteristics could be proved for the different genera or clusters.

9. Calicivirus infection has an important economical and social cost, not just due to the cost of foodborne outbreaks in adults but to the cost of sporadic cases affecting young children and causing severe disease.

10. Human caliciviruses are a relevant cause of illness in children, not just due to the number of affected children but due to the severity of the disease and the cost, economical and social, associated to this infection.

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8. <u>ANNEXES</u>

8.1. <u>RNA EXTRACTION</u>

<u>Material</u>

- Pipettes (Labsystems. Finnepipette 0.5 –10 ?L, Cat No. H70135; Finnepipette 5-50 ?L, Cat No. H71812; Finnepipette 50-200 ?L, Cat No. H74688; Finnepipette 100-1000 ?L, Cat No. H76773)
- Minicentrifuge (VWR Scientific Products. Model No.C-1200).
- Centrifuge (IEC.Centra MP4R)
- Vortex (Barnstead/Thermolyne. Maxi Mix II. Model No. M27615)
- Flat top microcentrifuge tubes 2.0 mL, polypropylene (Fisher Scientific. Cat No. 05-408-25A)
- Flat top microcentrifuge tubes 1.5 mL, polypropylene, (Fisher Scientific. Cat No. 05-408-10)
- 0.2-10 ?L pipette filter tips (Fisher Scientific. Cat No. 02-707-28)
- 1-50 ?L pipette filter tips (Tip One. USA Scientific, Inc, Cat No. 1120-2810)
- 1-200 ?L pipette filter tips (Tip One. USA Scientific, Inc, Cat No. 1120-8810)
- 100-1000 ?L pipette filter tips (Fisher Scientific. Cat No. 02-707-49)

<u>Reagents</u>

- Trizol (GibcoBRL Life Technologies. Cat No.15596-018).
- Chloroform (Sigma. Cat No. C-2432).
- Isopropanol anhydrous (Sigma. Cat No. 405-7).
- RNasin 40 U/?L (Promega. Cat No. N211A).
- NaCl (Sigma. Cat No. S-9888)
- KCl (Fisher scientific. Cat No. BP366-500)
- Na_2HPO_4 (Sigma. Cat No. S-0876)
- Dextrose (Baker Analized. Cat No. 1916-05)
- Tris-hidroxymethyl Aminoethanol (THAM)(Fisher scientific. Cat No. T395-5000).
- HCl 12 N (Fisher scientific. Cat No. A144-500).
- $MgCl_2$ –6 H_2O (Fisher scientific. Cat No. BP 214-500).
- $CaCl_2$ anhydrous (Sigma. Cat No. C-1016)

- Molecular biology grade water (Eppendorf. Cat No 00032-006159)

<u>Solutions</u>

Tris-buffered saline

NaCl	16 g
KCl	0.75 g
Na_2HPO_4	0.02 g
Dextrose	2 g
Tris-hidroxymethyl Aminoethanol (THAM)	6 g
Concentrated HCl 12 N	3.5 mL
$MgCl_2-6\,H_2O$	0.2 g
$CaCl_2$ anhydrous	0.2 g
Distilled water	1.87 L

Autoclave and aliquot under a hood

Store at -20^oC.

<u>Protocol</u>

Preparation of the samples

1. To prepare a 20% dilution in a 1.5 mL microcentrifuge tube labeled with the sample ID on top.

Depending on the type of sample:

o Solid stool sample: Suspend approx. 0.08 g of stool in 400 ?L of Tris-buffered-saline.

o Diarrhea stool sample: Suspend 80 µL of stool in 400 ?L of Tris-buffered-saline

o Diaper liner in liquid: Agitate the liner in the liquid then take 400 ?L

o Dry diaper liner: Add 500-1,000 µL of Tris-buffered-saline, agitate, then take 400 ?L of the liquid

o Extracts from diaper liner: transfer 400 ?L of the suspension

2. Vortex for at least 30 sec (or until the stool was completely suspended)

3. Keep the 20% dilution at 4ºC until the extraction

Extraction of RNA. Trizol method

1. Label 2.0 mL microcentrifuge tubes and put into each tube 1.0 mL of Trizol.

2. Label new 2.0 mL microcentrifuge tubes and put 900 ?L of isopropanol in each.

3. Vortex the 20% stool dilution for at least 30 sec (or until the stool was completely suspended).

4. Remove 400 ?L of the supernatant with a pipette (without touching the pellet) and put it in the tube with the Trizol.

5. Vortex for 30 sec and incubate at room temperature for 5 min.

6. Spin for 30 sec in the minicentrifuge to remove all the drops from the top.

7. Add 200 ?L of chloroform into each tube and vortex for 30 sec.

8. Incubate at room temperature for 3 min.

9. Spin at 12,000 rpm for 15 min at 4ºC

10. Remove 400 ?L of the clear phase with a pipette (without touching the interphase) and put the supernatant into the new tubes with isopropanol. Repeat this step until at least 800 ?L are in each tube.

11. Incubate the tubes at -20ºC for 1 hour.

12. Spin at 12,000 rpm for 15 min at 4ºC.

13. Remove all the isopropanol, allow the pellet to dry on a paper towel, tubes upside down.

14. Spin at 12,000 rpm for 1 min.

15. Remove the rest of the isopropanol with a pipette (do not touch the pellet or the side where it should be).

16. Leave the tube opened at room temperature for 5 min.

17. Prepare stock in a separate tube: 1 mL of molecular water and 10 ?L of RNasin (calculate the amount that you need for your samples).

18. Add 80 ?L of this water to each tube and mix it.

19. Store at -70º C

8.2. RT- PCR HUCV

Material

- Pipettes: Finnepipette. Labsystems (0.5 –10 ?L, Cat No. H70135; 5-50 ?L, Cat No. H71812; 50-200?L, Cat No. H74688; 100-1000 ?L, Cat No. H76773)
- Minicentrifuge C-1200
- Vortex (Maxi Mix II. Model No. M27615, Barnstead/Thermolyne).
- Flat top microcentrifuge tubes 2.0 mL, Polypropylene, Natural (Fisher Scientific. Cat No. 05-408-25A)
- Flat top microcentrifuge tubes 1.5 mL, Polypropylene, Natural (Fisher Scientific. Cat No. 05-408-10)
- Microcentrifuge tubes with attached caps 0.65 mL, VWR Scientific Cat No. 20170-293.
- 0.2-10 ?L pipette filter tips (Fisher Scientific. Cat No. 02-707-28)
- 1-50 ?L pipette filter tips (Tip One. USA Scientific, Inc, Cat No. 1120-2810)
- 1-200 ?L pipette filter tips (Tip One. USA Scientific, Inc, Cat No. 1120-8810)
- 100-1000 ?L pipette filter tips (Fisher Scientific. Cat No. 02-707-49)
- Syringe filter (Nalgene Cat No 190-2545)
- Programmable thermal controller (MJ Research, Inc, Model PTC-100)

Reagents

- Molecular biology grade water (Eppendorf Cat No. 00032-006159).
- Tris HCl (Fisher Scientific, Cat No. BP153-500)
- MgCl₂ (6 hidro) (Fisher Scientific Cat No. BP 214-500).
- KCl (Fisher Scientific Cat No. BP 366-500).
- Bovine serum albumin (BSA)
- Set of dATP, dCTP, dGTP, and dTTP (Promega Cat No. U1240).
- Rnasin Ribonuclease Inhibitor (40 U/µL) (Promega Cat No. N2111).
- AMV-Reverse Transcriptase (20 U/µL) (Promega Cat No. M9004).
- Primers (positive and negative sense) 0.1 mg/mL
- Taq
- Mineral oil (Sigma, Cat No. M-5904)

<u>Solutions</u>

10 x PCR buffer

	final concentration	molec weight	
Molecular bi ology grade water			up to 100 mL
TrisHCl (pH 8.5)	100 mM	157.6	1.576 g
MgCl$_2$ (6 hidro)	20 mM	203.3	0.406 g
KCl	500 mM	74.56	3.738 g

Autoclave in a big bottle firmly closed and with foil around the cap, filter, and aliquot under a hood.

Store at – 20°C

1%BSA

Autoclave 100 mL of purified water.

Mix with 1 g of bovine serum albumin.

Filter and aliquot under a hood.

Store at – 20 ^{o}C.

5 mM dNTP

Mix 400 µL dATP, 400 µL dCTP, 400 µL dGTP, and 400 µL dTTP.

Add 6.4 mL of molecular biology grade water.

Aliquot in 1.5 mL tubes and store at – 20°C.

<u>Protocol</u>

Reverse transcriptase reaction

1. Label n tubes
2. Mix the follow reagents (prepare for n+2 samples)

Samples	1		0	
Molecular biology grade water	13.7	µL	0	µL
10xPCR buffer	2.5	µL	0	µL
1% BSA	2.5	µL	0	µL
5mM dNTP mix	2	µL	0	µL
Negative sense primer (0.1 mg/mL)	1	µL	0	µL
Rnasin (40 U/µL)	0.1	µL	0	µL
AMV-RT (20 U/µL)	0.2	µL	0	µL
Total	22	µL	0	µL

3. Aliquot 22 µL into each tube

4. Add 3 µL of the purified RNA into each tube

5. Put the tubes into the thermocycler at 42°C for 1 hour (program RT)

6. Spin

PCR

1. Mix the follow reagents (prepare for n+2 samples)

Samples	1		0	
Molecular biology grade water	19.5	?L	0	?L
10xPCR Buffer	2.5	?L	0	?L
Positive-sense primer (0.1 mg/mL)	1	?L	0	?L
Taq (4-4)	2	?L	0	?L
total	25	?L	0	? L

2. Aliquot 25 µL into each RT tube mixing with the pipette

3. Add 2-3 drops of mineral oil into each tube

4. Firmly close the tubes

5. Put into the thermocycler (program C)

Program C:

Step 1: 94°C 3 min

Step 2: 94°C 1 min

Step 3: 49°C 1 min 30 sec

Step 4: 72°C 1 min

Step 5: Cycle step 2 to 4 for 39 times

Step 6: 72°C 15 min

Step 7: Hold at 4°C.

8.3. <u>VISUALIZATION OF RT-PCR PRODUCTS</u>

<u>Materials</u>

- Horizontal electrophoresis system (Horizon 2025 Bethesda Research Laboratories. Life technologies. Inc)
- Microwave.
- Magnetic stir plate: (Cimarec 2 Barnstead/Thermolyne. Model No SP46925)
- Magnetic stir bar
- Digital Camera (Kodak digital Camera DC 120: October 2000- June 2001; Kodak Digital Camera DC 290: June 2001-April 2002)
- Kodak 1D Image Analysis software (v.2.0 from Oct 2000 to Jun 2001, v.3.5 from August 2001-April 2002)
- UV transilluminator (Fisher Scientific. Model No FBTIV-B16)
- Pipettes: Finnepipette. Labsystems (0.5 –10 ?L, Cat No. H70135; 5-50 ?L, Cat No. H71812; 50-200 ?L, Cat No. H74688; 100-1000 ?L, Cat No. H76773)
- Natural pipet tips 1-200 µL (Tip One. USA Scientific, Inc. Cat No 1111-0700)
- Flat top microcentrifuge tubes1.5 mL, polypropylene (Fisher Scientific. Cat No. 05-408-10)
- Paraffin film (Parafilm "M". American can company)

<u>Reagents</u>

- Bromphenol blue (Sigma. Cat No. B-5525)
- Xylene cianol (Sigma. Cat No. X-4126)
- Glycerol (Fisher Scientific. Cat No BP229-1)
- Ethidium bromide (Sigma Cat No. E-8751)
- Water (purified)
- Tris crystallized free base (Fisher Scientific. Cat No BP152-500)
- Boric acid (Fisher Scientific. Cat No BP169-500)
- EDTA disodium salt (Fisher Scientific. Cat No BP120-500)
- Seakem LE Agarose (Biowhittaker Molecular Applications. Cat No 50004).

Solutions

Loading buffer (6X)

0.25% bromphenol blue

0.25% xylene cianol

30% glycerol in water

10 x TBE

Tris-base	108 g
Boric acid	55 g
0.5M EDTA	40 mL
or EDTA	9.3 g

Bring to a volume of 1 L with purified water. Store at room temperature.

Use 0.5 x TBE (100 mL of 10 x TBE in 2 L total volume)

Ethidium Bromide (10x)

1 g Ethidium bromide

100 mL purified water.

Stir until completely dissolved (1-2 hours).

Wrap in aluminum foil and store at -20°C.

Use 1x dilution

Protocol

1. Mix 300 mL of 0.5xTBE and 3 g of agarose in a beaker and heat up in a microwave for 8 minutes.

2. Allow to polymerize in the stirrer until the gel is warm.

3. Add 20 µL of ethidium bromide solution (1x) and pour it in the electrophoresis tray.

4. Leave at room temperature for about 1 hour.

5. Mix 2 µL of loading dye with 20 µL of sample using the paraffin film and load it into the agarose gel.

6. Put in the first and last well of each row 20 µL of a 1Kb marker.

7. Run for 1 hour at 175 V in a 0.5x TBE

Visualize in the UV transilluminator (using a filter) and take a picture.

8.4. <u>CLONING</u>

<u>Material</u>

- Pipettes: Finnepipette. Labsystems (0.5 –10 ?L, Cat No. H70135; 5-50 ?L, Cat No. H71812; 50-200 ?L, Cat No. H74688; 100-1000 ?L, Cat No. H76773)
- 0.1-10 ?L pipette tips
- 0.2-10 ?L pipette filter tips (Fisher Scientific. Cat No. 02-707-28)
- 1-50 ?L pipette filter tips (Tip One. USA Scientific, Inc, Cat No. 1120-2810)
- 1-200 ?L pipette filter tips (Tip One. USA Scientific, Inc, Cat No. 1120-8810)
- 100-1000 ?L pipette filter tips (Fisher Scientific. Cat No. 02-707-49)
- Microcentrifuge tubes with attached caps 0.65 mL, (VWR Scientific Cat No. 20170-293)
- Flat top microcentrifuge tubes 1.5 mL, polypropylene, (Fisher Scientific. Cat No. 05-408-10)
- Programmable thermal controller (Mastercycler gradient. Eppendorf scientific, Inc. Cat No 950-00-122-4)
- Water bath (Precision, microprocessor controlled 280 series, Cat No 51221046).
- Plastic petri dish 100x15 mm (Fisher Brand Cat No 05-757-12).
- Pipet-aid (Drummond Scientific)
- Serological pipette 50 mL (Costar Stripette, Corning, Inc. Cat No 4490).
- Polypropylene, V-bottom microplates (Multiplate 96. MJ Research, Inc. Cat No MLP-9601)
- Microseal film (MJ Research, Inc. Cat No MSA-5001)
- Culture tubes (Fisherbrand. Cat No 14-956-15)
- Cryogenic vials (Nalgene cryoware. Nalgene Brand Products. Cat No 5000-0020)
- Material for visualization of PCR products (see annex IV)

<u>Reagents</u>

- pGEM-T Vector System I (Promega. Cat No A3600)
- JM109 Competent cells (Promega. Cat No L2001)
- LB Agar, Lenox (Becton and Dickinson. Cat No 0401-17)

- LB Broth, Lennox (FisherBiotech. Cat No BP1427-500)

- Ampicillin sodium salt (Sigma. Cat No A-0166)

- IPTG (Sigma. Cat No I5502)

- X-gal (Molecular probes. Cat No 1690)

- Glycerol (Fisher Scientific. Cat No BP229-1)

- Reagents for PCR and visualitzation of PCR products (see annex III and IV)

Protocol

Ligation

Keep everything on ice and thaw buffer completely

1. Mix the following reagents

	1	sample		x	samples
T4 DNA Ligase 2X Buffer	5	μL			
pGEM-T Vector (50 ng)	1	μL			
T4 DNA Ligase	1	μL			
PCR product	3	μL			

2. Incubate overnight at 4^oC (or 2 hours at 16^oC)

3. Heat reactions for 10 min at 70-72°C, allow cooling at room temperature (store at -20°C).

Transformation

1. Prepare the plates: Mix 1 L of purified water with 35 g of LB agar. Autoclave and allow it to cool to aprox 45°C. Add 1 mL of ampicillin, 1 mL of IPTG, and 1 mL of X-gal. Mix and pour in the plastic petri dishes (about 15 mL per plate). Let them solidify.

2. In a 0.65 mL Ependorf tube put 2 μL of the ligated PCR product-pGEM-T vector and add 40-50 ? L of competent bacteria.

3. Keep on ice for 20 min.

4. Heat at 42°C for 45 sec.

5. Add 400 μL of LB if the product is short (less than 400 bp) and 300 μL of LB if the product is long (more than 500 bp).

6. Shake at 20 rpm and 37°C for 30-60 min.

7. Seed onto the plate about 200-300 μL

8. Incubate at 37°C overnight

Screening of the clones

1. Mix the following reagents

Number of Samples	1		0	
PCR Mix				
BSA (1%)	1.25	µL	0.00	
10X PCR Buffer	2.5	µL	0.00	
Water	18.75	µL	0.00	
150 mM dNTP	1	µL	0.00	
R primer 0.1µg/µL	0.5	µL	0.00	
F primer 0.1µg/µL	0.5	µL	0.00	
Taq (4/4)	0.5	µL	0.00	
Add to each tube	25	*ul*	25.00	

2. Aliquot 25 µL in each multiplate well.

3. Using a pipette transfer one colony to each well

4. Place the multiplate in the thermocycler (program Screen20)

    Screen20:

    Step 1: 94ºC 3 min

    Step 2: 94ºC 1 min

    Step 3: 49ºC 1 min 30 sec

    Step 4: 72ºC 1 min

    Step 5: Cycle step 2 to 4 for 20 times

    Step 6: 72ºC 15 min

    Step 7: Hold at 4ºC.

5. Visualize the PCR products (see protocol RT-PCR).

Growing the selected clones

1. Prepare 4 mL of LB with ampicillin (1 µL of ampicillin/mL of LB broth) per clone.

2. Put the selected clone into the LB (use a pipete)

3. Put in the shaker overnight (20 rpm and 37°C)

8.5. <u>PURIFICATION OF PLASMID DNA</u>

<u>Material and reagents</u>

- Centrifuge (IEC.Centra MP4R)
- Vortex (Barnstead/Thermolyne. Maxi Mix II. Model No. M27615)
- Pipettes (Labsystems. Finnepipette 5-50 ?L, Cat No. H71812; Finnepipette 50-200 ?L, Cat No. H74688; Finnepipette 100-1000 ?L, Cat No. H76773)
- 1-50 ?L pipette tips (Tip One. USA Scientific, Inc, Cat No. 1120-2810)
- 100-1000 ?L pipette filter tips (Fisher Scientific. Cat No. 02-707-49)
- Flat top microcentrifuge tubes 2.0 mL, polypropylene (Fisher Scientific. Cat No. 05-408-25A)
- Flat top microcentrifuge tubes 1.5 mL, polypropylene, (Fisher Scientific. Cat No. 05-408-10)
- QIAprep Spin Miniprep kit (Qiagen Cat No 27142)

<u>Protocol</u>

1. Put 2 mL of the culture in a tube 2.0 mL microcentrifuge tube.
2. Spin at 12.000 rpm for 1 min and remove the supernatant (decant)
3. Add the rest of the culture and spin at 12,000 rpm for 1 min
4. Remove the supernatant and spin it again for 1 min.
5. Remove the rest of the supernatant with a pipette
6. Add 250 ?L of the P1 buffer (ensure that the RNase has been added) and vortex (no cell clumps should be visible).
7. Add 250 ?L of P2 buffer and mix gently by inverting the tube until the solution becomes viscous and slightly clear (do not vortex). Do not allow the lysis reaction to proceed for more than 5 min.
8. Add 350 ?L of N3 buffer (neutralizing buffer) and invert the tube immediately but gently 4-6 times.
9. Spin at 12,000 rpm for 10 min
10. Pour the supernatant in a new tube
11. Spin at 12,000 rpm for 5 min

12. Place a QIA prep column in a 2 mL collection tube.

13. Pour the supernatant of the step 15 in the column kit and spin for 1 min.

14. Discard the flow-through.

15. Add 500 ?L of PB buffer in the spin column.

16. Spin for 1 min and discard the flow-through.

17. Add 750 ?L of PE buffer (ensure the ethanol has been added).

18. Centrifuge for an additional 1 min to remove residual wash buffer.

19. Place the spin column in a new 1.5 mL microfuge tube.

20. Add 50 ?L of Buffer EB to the center of each QIAprep column and let stand for 1 min.

21. Spin for 1 min at 12,000 rpm.

8.6. <u>SEQUENCING</u>

<u>Materials and reagents</u>

- Pipettes: VWRbrand 0,5-10 µL, Cat No. 89-400498, Finnepipette. Labsystems 5-50 ?L, Cat No. H71812)
- Minicentrifuge C-1200
- Vortex (Maxi Mix II. Model No. M27615, Barnstead/Thermolyne).
- 0.1-10 ?L pipette tips (Tip One. USA Scientific. Inc. Cat No. 1111-3700)
- 1-50 ?L pipette filter tips (Tip One. USA Scientific, Inc. Cat No. 1120-2810)
- Microcentrifuge tubes with attached caps 0.65 mL, VWR Scientific Cat No. 20170-293.
- Polypropylene, V-bottom microplates (Multiplate 96. MJ Research, Inc. Cat No MLP-9601)
- Microseal film (MJ Research, Inc. Cat No MSA-5001)
- Molecular biology grade water (Eppendorf Cat No. 00032-006159).
- SequiTherm EXCEL II Long-Read DNA Sequencing Kit-ALF (Epicentre technologies. Cat No SE8301A).
- ReproGel for polyacrylamide gel electrophoresis (Amersham Pharmacia biotech. Cat No 17-6001-09)
- UVA-lamp (ReproSet. Amersham pharmacia biotech. Cat No. 16-1125-64)
- Sequencer (ALFexpress DNA sequencer v2.0. Amersham Pharmacia. Cat No 18110785)
- ALFwin Sequence Analyser v2.0. Amersham Pharmacia

<u>Protocol</u>

1. Keep all the reagents on ice.
2. Prepare a microsample plate with 4 rows and 10 columns. Label the first row with A, the second with C, the third with G, and forth with T
3. Vortex the ddNTP. Distribute 2 ?L of mix A into each well of the first row, 2 ?L of mix C in each well of the second row, 2 ?L of mix G in the third row, and 2 ?L of mix T in each well of the fourth row.

4. Mix (for each sample to sequence)

> Seq Buffer   7.2 ?L
>
> Primer (2 pmol)   1 ?L
>
> Template + H$_2$0   until 16 ?L
>
> Taq polymerase   1 ?L

5. Dispense 4 ?L of the DNA mix to the microsample plate. Mix thoroughly.

6. Cover with microseal film and place in the termocycle.

7. Program Seq (1.5 hr).

> Step 1: 95°C, 5mins
>
> Step 2: 95°C, 30s
>
> Step 3: 50°C, 15s
>
> Step 4: 70°C, 1min
>
> Step 5: Repeat 29 times from step 2 to 4.
>
> Step 6: Hold at 4°C

8. Add 3 ?L of loading dye to each reaction and mix by gentle agitation.

9. Heat the samples 5 min at 90°C in the thermocycler before loading

10. Leave on ice.

11. Cast the gel (pour the mix gel solution along the open slit between the glass plates at the lower edge of the gel cassette and place it under the UVA lamp for 10 min).

12. Run Set-Up-ALF-express Software

13. Open and save Casebook with new sample information and conditions of the reaction (run time: 650 min, voltage: 1500 V, current: 60 mA, power: 25, temperature: 55°C, sample interval: 2 s)

14. Load 6 ?L of each sample into the gel.

15. Preset, and check parameters (water level, temperature, laser intensity).