

**Comparative molecular genetic studies of nucleic
acid detection in human noroviruses**

Ph.D. Thesis

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Summary

Introduction: The public health impact of HuCV infections is increasingly recognized. Noroviruses are the commonest cause of outbreaks of non-bacterial gastroenteritis, the most commonly recognized foodborne viral infection and second only to rotavirus as a cause of severe diarrhea in children. The key factors underpinning this high burden of infection are their low infectious dose (10–100 virus particles are enough to infect), their stability in the environment (acids, pH, chloride and temperature), the wide diversity of strains and the lack of any long-term immunity to an infection or illness.

The recent development of sensitive real-time RT-PCR tests for diagnosis, quantification and characterization of these agents has led to the recognition of the importance of norovirus infection. Evidence suggests that the detection of noroviruses in fecal specimens by conventional and real-time RT-PCRs may be limited by factors such as low virus concentrations in feces, improper specimen storage, inefficient viral RNA extraction, the presence of fecal reverse transcriptase inhibitors and the use of different primers. In addition, noroviruses are genetically extremely diverse and none of the reported conventional and real-time RT-PCR assays are able to detect all strains.

Aims: The aims of this study were to find the most effective nucleic acid isolation method for an effective norovirus diagnosis, to introduce the real-time RT-PCR assay for time-saving and for a more sensitive diagnosis. For the rapid detection of norovirus antigen in stool samples an ELISA method was introduced and it was compared with two commercial available RT-PCR kits. Both RT-PCR techniques and ELISA systems are very important to clarify the pathogenic role of noroviruses in sporadic cases and in epidemics in Szeged and in its catchment area.

Results:

1. Five different RNA purification methods were compared.
2. 30 samples were compared by using the traditional method and the two-step real-time PCR method (by using the Jiang-designed primers). 23 (76.7%) samples were positive by using the real-time RT-PCR and only 6 (20%) samples were positive with the traditional RT-PCR.
3. On using the two-step real-time RT-PCR, we found that 38 (9.92%) of the 383 samples contained HuCV. 14 (10.07%) of the 139 samples proved positive for HuCV on the use of the one-step real-time RT-PCR method by the Jiang-designed primers. First we compared the Jiang-designed and our newly-designed primers on 110 samples by using traditional RT-PCR. We found that 32 (29.1%) samples

were positive by using our newly designed primers, but negative with the Jiang-designed ones. Secondly, 66 samples were compared by the two two-step real-time RT-PCR method. 12 (18.1%) proved to be positive with the newly-designed primers and only 4 (6.06%) were positive with the Jiang-designed ones.

4. The IDEIATM Norovirus ELISA revealed 38 norovirus-positive and 23 negative samples. The sensitivity of the test was 78.9%, the specificity was 100%, the PPV was 100% and the NPV was 39.1%. By using the Argene Calici/Astrovirus Consensus kit of the 61 samples, 48 were positive for HuCVs, 10 were negative, 2 were borderline and 1 contained inhibitors. The sensitivity of the test was 92.8%, the specificity was 100%, the PPV was 100% and the NPV was 69.2%. With the Cepheid Norovirus Primer and Probe Set, 47 of the 61 stool samples proved to be positive for human noroviruses; 46 were GGII and only 1 was GGI-positive. 8 samples were negative and only 6 contained inhibitors. The sensitivity of the test was 91.2%, the specificity was 100%, the PPV was 100% and the NPV was 64.3%.
5. Between 1 January 2004 and 31 March 2007, 1,152 stool samples were collected from children in the age group between 0 and 3 years. Of the overall 1,152 stool samples, 187 (16.2%) proved positive for noroviruses. Between 2003 and 2011, 5,031 stool samples were examined for human noroviruses. 836 (16.6%) proved to be positive for noroviruses. In the 9-year-period, 10 norovirus accumulations were observed at the different units of the Albert Szent-Györgyi Clinical Center.

Conclusion: During the study, we compared five different RNA purification method, we showed their benefits and disadvantages. We successfully developed first a two-step real-time SYBR Green RT-PCR assay for the norovirus diagnostics, and then translated it into one-step real time RT-PCR. We have developed a primer pair (targeting the RNA-depending RNA polymerase region), with which the norovirus diagnostics have become safely practicable in the European region. During our study, we compared two commercial available RT-PCR kits and one antigen-ELISA kit and found that this antigen-ELISA kit is a very good screening kit, with which the accumulations have become detectable. For genetic analyses and for sporadic cases the RT-PCR is the gold-standard method. By using this commercial available kit we can differentiate between GGI and GGII without sequencing the PCR product. In the past 9 years we investigated the role of noroviruses in sporadic cases and also in accumulations in the different hospital wards.

List of Abbreviations

DMSO	Dimethyl sulfoxide
EIA	Enzyme immuno assay
ELISA	Enzyme-linked immuno assay
EM	Electron microscopy
ER	Endoplasmic reticulum
GGI	Genogroup I
GGII	Genogroup II
HBGA	Histo-blood group antigen
HuCV	Human calicivirus
IEM	Immuno electron microscopy
LC	Light Cycler
NA	Nucleic acid
NLV	Norwalk-like viruses
NPV	Negative-predictive value
ORF	Open reading frame
PPV	Positive-predictive value
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNasin	RNase inhibitor
RT-PCR	Reverse transcription polymerase chain reaction
SLV	Sapporo-like viruses
SPC	Sample preparation control
Tm	Melting temperature

I. General characteristics of noroviruses

I.1. Discovery of human caliciviruses, taxonomy and genetic classification

The syndrome associated with caliciviral gastroenteritis was described in the medical literature over 70 years ago. However, it was many years later that a causative agent could be linked with the condition Zahorsky described in 1929 as “winter vomiting disease”⁽¹⁾.

Studies performed in Ohio in the late 1940s demonstrated just how much gastroenteritis could not be attributed to known bacterial or parasitic pathogens. All of the cases ascertained over a 30-year period 75% had no adequate explanation⁽²⁾. These were said to present acute, infectious and non-bacterial gastroenteritis. Clinical studies where volunteers were exposed to fecal extracts that had been filtered to remove all bacteria, confirmed the hypothesis that a viral agent was likely cause⁽³⁾.

In the autumn of 1968, 50% of the students and teachers in an elementary school in the town of Norwalk, were struck with an illness characterized principally by nausea, vomiting and abdominal pain. Since no bacterial agent was found, a viral case was suspected. However, because these viruses did not grow in tissue culture, no causative agent could be recovered⁽⁴⁾.

In 1972 Kapikian discovered the etiology of the virus. By IEM examinations, the Norwalk virus, the prototype agents of the genus *Norovirus* (previously called “Norwalk-like viruses”) was identified⁽⁵⁾. Several other viral causes of gastroenteritis, most notably rotavirus and adenoviruses, were elucidated in the 1970s by the same technique.

The family *Caliciviridae* is composed of small (27 to 40 nm), nonenveloped, icosahedral viruses that possess a linear, positive-sense, single stranded RNA (ssRNA+) genome.

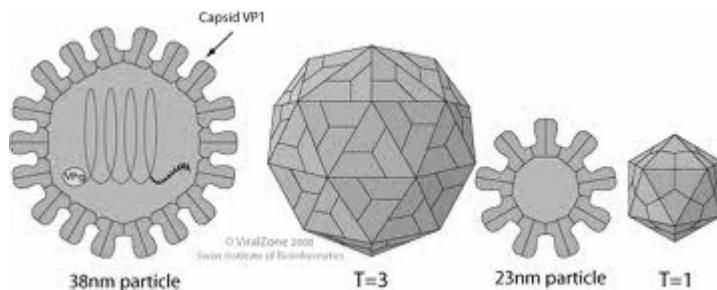


Figure 1. Non-enveloped, icosahedral with T=3 symmetry, about 38–40 nm in diameter.. Small empty virions are about 23nm in diameter, and would be of icosahedral T=1 symmetry (Source: Viral Zone, http://viralzone.expasy.org/all_by_species/32.html)

The four genera of the family are: *Norovirus*, *Sapovirus*, *Vesivirus* and *Lagovirus*. *Vesivirus* and *Lagovirus* are important veterinary pathogens. The major medical human pathogens in the family are noroviruses and sapoviruses. A standardized nomenclature was proposed to classify noroviruses into 29 genetic clusters that fall within five genogroups. Most of the strains relevant to the human disease belong to genetic clusters within GGI and GGII⁽⁶⁾ (Table 1).

Genogroup	Host	No. of sequences	Clusters	New clusters
G1	Human	30	8	1
G2	Human/Porcine	121	17	5
G3	Bovine	9	2	
G4	Human	3	1	
G5	Murine	1	1	
Total	5	164	29	6

Table 1. Genogroups and clusters of norovirus strains⁶

I.2. Physicochemical properties of human caliciviruses

Norwalk virus (genus *Norovirus*) has a reported buoyant density of 1.33 to 1.41g/cm³ in cesium chloride. The Norwalk virus retains infectivity for volunteers following a) exposure of the stool filtrate to pH 2.7 for 3 hours at room temperature, (b) treatment with 20% ether at 4°C for 18 hours, or (c) incubation at 60°C for 30 minutes. Norwalk virus is resistant to inactivation following its treatment with 3.75 to 6.25mg/L of chlorine (free residual chlorine of 0.5 to 1.0mg/L), a chlorine concentration consistent with that found in a drinking water distribution system. However, Norwalk virus is inactivated following treatment with 10mg/L chlorine⁽⁷⁾.

1.3. Genome organization of Noroviruses

Noroviruses contain a positive-sense ssRNA genome of 7,642 nucleotides; at the 3' end polyadenilated tail is located. The norovirus genome consists of a single strand of positive-sense RNA organized into 3 open reading frames (ORFs). ORF1 encodes nonstructural proteins such as RNA dependent RNA polymerase, ORF2 encodes viral capsid protein 1, and ORF3 encodes a small capsid protein (viral capsid protein 2) associated with stability of viral capsid protein 1. The virus particles demonstrate an amorphous surface structure when visualized using electron microscopy and are between 27–38 nm in size⁽⁷⁾.



Figure 2. Norovirus genome structure

1.4. Clinical manifestations and management of the norovirus infection

In the absence of other factors, infections with noroviruses are typically mild and self-limiting diseases. Norovirus illnesses can present relatively severe symptoms of vomiting and non-bloody diarrhea, with the symptoms usually resolving in 2 to 3 days. Several studies have shown that the median duration of the illness can be longer (4-6 days) in patients affected during hospital outbreaks and in children <11 years of age^(8,9). Vomiting is relatively more prevalent in persons >1 year of age, whereas children <1 year more often develop diarrhea⁽⁸⁾. Fever, which is reported in 37–45% of the patients, typically resolves within 24h. Sensitive diagnostic assays have detected noroviruses in stool for up to 3 weeks in patients with either symptomatic or asymptomatic infection.

Oral rehydration solutions that provide essential electrolyte replacement plus sugar (glucose or sucrose) may be administered as first-line therapy for uncomplicated diarrheal illnesses. Patients presenting with signs and symptoms of significant dehydration and those unable to tolerate oral fluids may require early parenteral fluid plus electrolyte replacement. As food

tolerated, patients could begin taking food early in the illness since adequate caloric intake might enhance patient recovery.

I.5. Pathogenesis and immunity

Noroviruses are contracted by humans via the oral route. As they are acid-stable viruses, they pass through the stomach; replication occurs in the small intestine. Most of our knowledge concerning the pathogenesis of noroviruses comes from volunteer studies performed in the USA. Light and EM showed that individuals with clinical illness exhibit lesions on the small intestine mucosa. The mucosa lining becomes inflamed and absorptive epithelial cells develop an abnormal appearance. Blunting of the villi, shortening of the microvilli, dilatation of the ER, swollen mitochondria, and intracellular edema are also observed microscopically. Within 2 weeks, the small intestine returns to a normal histological appearance.

After a norovirus infection, there is some short-term immunity to noroviruses; long-term immunity does not appear to be conferred by a single infection. Recent research suggest, that host genotype is a prominent factor in the development of norovirus infection since norovirus infection depends on the presence of specific human histo-blood group antigen receptors in the gut of susceptible hosts⁽¹⁰⁾. The combination of the strain specific binding and the variable expression of the HBGA receptors may explain the varying host susceptibility observed in norovirus outbreaks and volunteer studies.

There is also some evidence that people with blood types B and AB may be partially protected against symptomatic infection, but those with blood group O may be at greatest risk of severe infection.

I.6. Diagnosis of noroviruses

I.6.1. Electron microscopy

Since cell culture systems for noroviruses have not been developed, EM has been a fundamental tool used by the investigators. Samples are prepared for EM by a simple and inexpensive negative staining technique. Direct detection of noroviruses by EM is only possible in samples with a viral load more than 10^6 particles/ml⁽¹¹⁾. These enteric viruses can only be detected for approximately 48 hours after the onset of the symptoms. In IEM, stool samples are visualized after reaction with antibody derived from convalescent-phase sera from infected individuals with gastroenteritis⁽¹²⁾. Antigen and antibody form immune-

complexes, which can be negatively stained. IEM was used by Kapikian in the discovery of the Norwalk agent (Figure 3).

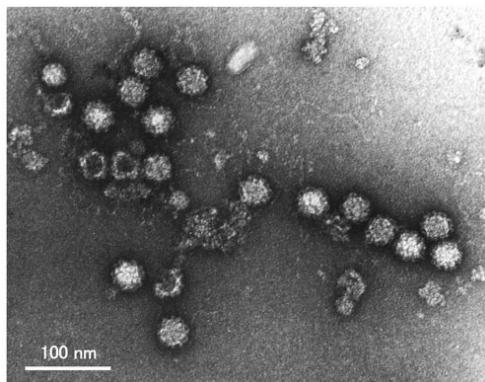


Figure 3. Norovirus GI/4 Chiba407 strain visualized by electron microscopy

(Source: <http://www.glycoforum.gr.jp/science/glycomicrobiology/GM02/GM02E.html>)

I.6.2. Immunological studies

There are several norovirus antigen detecting EIAs using monoclonal antibodies (native baculovirus-expressed proteins). While these assays are highly sensitive compared with EM or IEM, their use in diagnostic laboratories has been limited by their narrow specificity (only norovirus GGI and GGII). The detection limit for ELISA assays is currently estimated at 10^4 to 10^5 particles per ml.

I.6.3. Molecular biological techniques

Amplification of the Norwalk virus by RT-PCR was first achieved by Jiang et al. in 1992⁽¹³⁾ and has since become a common diagnostic and research tool worldwide. The complete sequencing of a range of human caliciviruses has led to the development of many primer pairs for use in RT-PCR. In comparison to EM, RT-PCR is a far most sensitive diagnostic tool and able to detect virus for 2 weeks after the infection and possibly longer⁽¹⁴⁾. Due to the high genetic diversity, it has been difficult to find an appropriately sensitive and specific primer pair to detect all noroviruses. Using sequence information of an increasing number of Norovirus strains, several research groups successfully developed RT-PCR assays based on improved primers targeting the POL gene (region A)^{15,16,17,18,19,20}. Subsequently, different primer sets targeting region A have been used successfully in epidemiological studies for the diagnosis of Norovirus in fecal specimens from both outbreaks and sporadic cases^{15,16,21,22,23}.

In addition, primers directed to other regions of the Norovirus genome have been developed including relatively conserved regions at the 3'-end of ORF1 (region B^{24,25,26}), at the 5'-end of ORF2 (region C and E;^{18,27,28}) and at the 3' end of ORF2 (region D²⁹) (Figure 4).

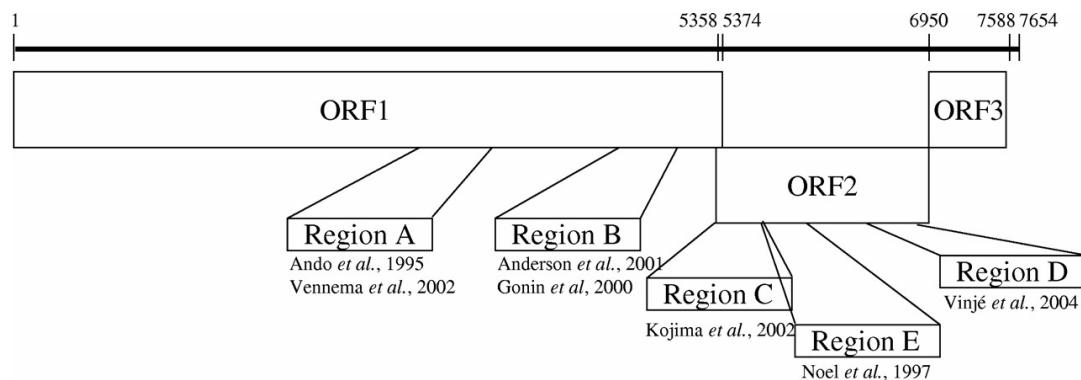


Figure 4. Schematic presentation of a norovirus genome and positions of regions (A–E) commonly used for detection and genotyping³⁰

Real-time PCR systems are excellent diagnostic tools; they are more specific and sensitive than traditional PCRs. Real-time PCR was developed for the production and quantification of amplicons using intracellular dyes or fluorescent probes or primers.

I.6.4. Epidemiological methods

Laboratory confirmation has not been possible for the majority of outbreaks. Even today, the etiology of many outbreaks goes unconfirmed because the sensitive ELISAs and RT-PCRs are not widely available outside reference laboratories and because appropriate samples are not always collected.

A set of criteria proposed by Kaplan *et al.*⁽³¹⁾ stipulates that an outbreak can be attributed to a viral case if:

- stool cultures are negative for bacterial pathogens;
- mean incubation period is 24–48 hours;
- mean duration is 12 to 60 hours and
- there is vomiting in $\geq 50\%$ of cases.

1.7. Seasonality, age distribution and seroprevalence

Norovirus infection is prevalent seen during the colder months of the year. In European settings, it begins to increase in October or November, peak around January, and tails-off by May or June.

Norovirus infections can occur at any time. The highest incidence of norovirus infection is in children under 5 years of age and, among children, the commonest cause of gastroenteritis is viral, with noroviruses being at least as frequent as rotavirus⁽³²⁾. It is very important to note, that a large number of norovirus outbreaks are reported from nursing homes with elderly residents. The attack rates are only slightly lower among staff than among elderly residents⁽²³⁾. In a survey in England, Gray et al. found that nearly three quarters of those tested had norovirus antibody. Antibody prevalence was highest among the middle-aged and the elderly; at every age-group 30 years or older, antibody prevalence was nearly 90%. Prevalence was also high among infants (<6 months old), at 75%. This is likely a measure of maternal antibodies. Among the 6-11 months age-group, antibody prevalence was 25%, this rose through adolescence and young adulthood⁽³³⁾. A very similar pattern was found in Sweden, with an overall prevalence of approximately 80%⁽³⁴⁾.

1.8. Norovirus vaccines

Without the ability to grow the norovirus in cell cultures, the researchers turned to insert a human norovirus capsid gene – capsid refers to the virus's outer shell – into a specific location on the genome of a different virus. This process creates what is known a recombinant virus – a new viral strain formed by recombining genetic material from other viruses. The viral host for this vaccine candidate is called vesicular stomatitis virus, or VSV. Animals receiving the vaccine developed high levels of antibodies, a robust white blood cell response and an additional immune response in the area of the body most affected by this particular infection – the gastrointestinal system. VSV-based recombinant is also considered a powerful application because it can be essentially used as a bioreactor to facilitate large-scale production of these specific virus-like particles. In addition, it saves time: the viral vector developed virus-like particles within two days.

Because mice will not develop traditional norovirus symptoms, this study did not involve a test of the vaccine against the pathogen itself. The researcher hopes to test the vaccine candidate in a larger animal model, such as germ-free pigs, animals that have never been

exposed to any pathogens. These animals develop diarrhea in response to norovirus infection, as do humans⁽³⁵⁾

I.9. Transmission of noroviruses

Noroviruses cause outbreaks through a number of well-documented transmission routes including person-to-person, foodborne and waterborne routes (Figure 5). Recently, the roles of environmental contamination and contamination of raw fruit and vegetables have been demonstrated. Person-to-person transmission has been documented in two ways, fecal-oral and aerosol formation following vomiting.

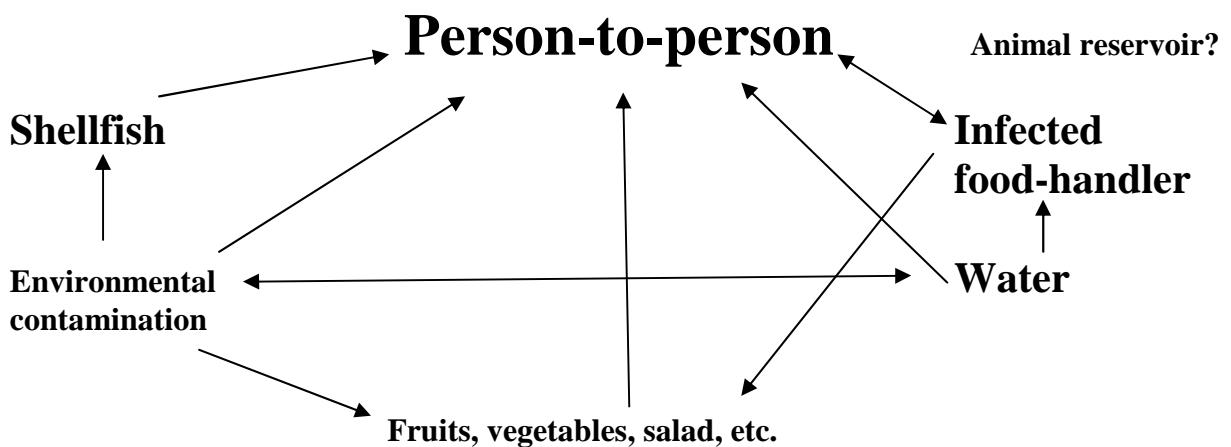


Figure 5. Transmission routes of noroviruses. The relatively large font size of the “person-to-person” sign represents the current understanding of its importance as the dominant mode of transmission. Other routes, such as the environmentally contaminated shellfish, may seed wide epidemics, introduce new strains to an area or cause infection multiple strains-providing the right circumstances for genetic recombination occur. Frequently, outbreaks are not exclusively spread by one route, moreover, attributing an outbreak to a single mode of transmission is somewhat arbitrary⁽³⁶⁾

II. Aims of the study

This study was carried out with the following aims:

- II.1. For the human calicivirus, RT-PCR becomes necessary to find the most effective nucleic acid isolation method for an effective diagnosis: traditional Trizol-based extraction (Trizol-Genetron, TRI-Reagent) was compared with the viral RNA isolation by using commercial kits (V-Gene Total RNA Preparation Kit, V-Gene Viral RNA/DNA Preparation kit, Roche Total RNA Preparation Kit).
- II.2. A time-saving and the more sensitive real-time RT-PCR technique was introduced for comparison of the traditional RT-PCR and the two-steps real-time RT-PCR techniques.
- II.3. By working with the Jiang-designed primers we found many aspecific products, for example: 11th human chromosome fragment, astroviral RNA or enteroviral RNA. To decrease the aspecific products in clinical samples, we decided to create a new primer pair, with which the norovirus detection would be more specific.
- II.4. For the detection of norovirus antigen in stool samples (comparison of ELISA-based methods and different commercial PCR kits), an ELISA method was introduced.
- II.5. We decided to clarify the pathogenic role of noroviruses in sporadic cases and in epidemics in Szeged and in its vicinity by using the RT-PCR techniques and ELISA systems.

III. Patients and methods

III.1. Sample collection and processing

Stool samples. Samples were collected from infants, children and adults (from the pediatric patients of family doctors and from eight different wards at the University of Szeged) with clinical symptoms of acute gastroenteritis: nausea, vomiting and/or three or more loose stools in 24 hours. Exclusion criteria: rotavirus, adenovirus or other enteric positivity. One stool sample was collected per patient during the acute phase of the infection; samples were stored at +4°C until processing. After examination, samples were stored at -20°C. A stool suspension from 10% to 50% in 1 ml sterile PBS was performed. The sample was centrifuged at 12,000g for 15 minutes. The supernatant was collected and was used for the RNA extraction.

III.1.1. Trizol-based methods: Trizol-Genetron method and TRI Reagent (Sigma, Saint Louis, USA)

This protocol was performed for the purification of viral RNA from 150µl stool sample. Through many lysis, centrifugation and pipetting steps, viral RNA was precipitated to the wall of the Eppendorf tube. Viral RNA was reconstituted in 20–40µl RNA-free water. Extracted viral RNA was stored at -70°C.

III.1.2. V-Gene Total RNA and V-Gene viral RNA/DNA Preparation kit

a) V-Gene Total RNA Preparation kit (Building B2-1, Xiacheng Industrial Zone (Huafeng, Shiqiao), Hangzhou 310022, P.R. China)

Viral RNA was extracted according to the manufacturer's instructions. Different from the conventional method, hazardous reagent such as phenol, chloroform or ethidium bromide is not used in this Kit. This protocol is performed for the purification of viral RNA from 100µl stool sample. Briefly, 5 buffers are incorporated in the kit: a cell lysis buffer (R-A), a protein removing buffer (R-B), two phase-separating buffers (R-C and R-D), a RNA extrication buffer (R-E) and a TE buffer for the RNA reconstitution. Through many lysis and centrifugation steps, viral RNA was precipitated to the wall of the Eppendorf tube. Viral RNA was reconstituted in 50µl buffer. Extracted viral RNA was stored at -70°C.

b) V-Gene viral RNA/DNA Preparation Kit (Building B2-1, Xiacheng Industrial Zone (Huafeng, Shiqiao), Hangzhou 310022, P.R. China)

Viral RNA was extracted according to the manufacturer's instructions. This protocol is performed for the purification of viral RNA from 50–160 μ l stool sample. Briefly, 6 buffers were incorporated in the kit: a viral lysis buffer (G-AV), a phase-separating buffer (G-BV), a nucleic acid binding buffer (G-CV), two wash buffers (W1 and W2) and an elution buffer (2.5 mM Tris-HCl, pH 8.5 RNA/DNA free). Buffer G-AV lyses all sorts of viral particles and releases viral RNA/DNA. Proteins, dyes, lipids and other impurities that inhibit PCR were separated from viral RNA/DNA by a unique two-phase partition. Highly purified viral nucleic acid in the lower phase was then recovered by binding to silica membrane in the presence of high concentration of chaotropic salt. After the washing steps the purified viral nucleic acid on the membrane was then eluted in low-salt Tris buffer or water. Viral RNA was eluted from the column with 40 μ l elution buffer. Extracted viral RNA was stored at -70°C.

III.1.3. Roche Total RNA Preparation kit (Roche Diagnostics GmbH, Mannheim, Germany)

Viral RNA was extracted according to the manufacturer's instructions. This protocol is performed for the purification of viral RNA from 200 μ l stool sample. Briefly, 4 buffers are incorporated in the kit: a binding buffer, an inhibitor removal buffer, a wash buffer and an elution buffer. Nucleic acids (NA) bind to the surface of glass fiber fleece in the presence of chaotropic salt. This allows the High Pure filter tube to specifically immobilize nucleic acids (both DNA and RNA) while they are freed from contaminants. Bound nucleic acid was washed two times and the purified RNA was eluted in 50 μ l elution buffer. Extracted viral RNA was stored at -70°C.

III.1.4. QIAamp Viral RNA Mini kit (Qiagen, Victoria, Australia, Ref. No. 67-120A)

QIAamp Viral RNA Mini Kits represent a well-established general-purpose technology for viral RNA preparation. The kit combines the selective binding properties of a silica-gel-based membrane with the speed of microspin or vacuum technology and is ideally suited for simultaneous processing of multiple samples. The sample is first lysed (Buffer AVL) under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions are then adjusted to provide optimum binding of the RNA to the QIAamp membrane, and the sample is loaded onto the QIAamp spin column. The RNA binds to the membrane, and contaminants are efficiently washed away in two steps using two

different wash buffers (Buffer AW1 and AW2). High-quality RNA is eluted in a special RNase-free buffer (Buffer AVE), ready for direct use or safe storage. The purified RNA is free of protein, nucleases, and other contaminants and inhibitors. The special QIAamp membrane guarantees extremely high recovery of pure, intact RNA in just twenty minutes. This protocol is performed for the purification of viral RNA from 140 μ l stool sample, the elution volume was 60 μ l. Extracted viral RNA was stored at -70°C.

III.2. Traditional PCR (RT-PCR, agarose gel-electrophoresis)

Primers were as described by Jiang et al. in 1999⁽¹⁹⁾: the HuCV sense primer p289, 5'-TGACAATGTAATCATCACCATA (nt position: 4865–4886) and the antisense primer p290/a, which has not been published. The sequence of our new primer pair was: 5'-CCCTAGAAATCATGGT-3' (nucleotide position 46–60) and 5'-CCAGTGGGCGAT-3' (nucleotide position 196–185).

Reverse-transcription. The total amount of RT mix/sample was 47.5 μ l, which contained the following components: 0.14U/ μ l M-MuLV-Reverse Transcriptase (Fermentas GmbH, St. Leon-Rot, Germany); 0.2U/ μ l rRNasin (Fermentas GmbH, St. Leon-Rot, Germany); 3ng/ μ l p289 (synthesized in the Biological Research Centre, Hungarian Academy of Sciences, Szeged Centre of Excellence of the European Union); 10 mM dNTP; 0.5mM MgCl₂ (Sigma, Saint Louis, USA); 10xPCR Buffer [100 mM Tris-HCl (pH: 8.3), 15mM MgCl₂, 500mM KCl, 0.01% gelatine Sigma, Saint Louis, USA]; and RNase free water (Promega, Madison, USA). 3 μ l RNA was added to the RT mix, after which the mixture was incubated at 42°C for 1 hour. Phosphate-buffered saline and known HuCV-positive specimens were used as negative and positive controls.

PCR. The PCR master-mix contained the following components: RNase free water (Promega, Madison, USA); 10x PCR Buffer [100 mM Tris-HCl (pH: 8.3), 15mM MgCl₂, 500mM KCl, 0.01% gelatine; Sigma, Saint Louis, USA]; 0.5mM MgCl₂ (Sigma, Saint Louis, USA); 0.1U/ μ l FastStart Taq DNA Polymerase (Roche Diagnostics GmbH, Mannheim, Germany) and 3ng/ μ l p290/a (synthesized in the Biological Research Centre, Hungarian Academy of Sciences, Szeged Centre of Excellence of the European Union). 50 μ l PCR master mix was added to the above mentioned RT mix and used the following PCR program with the Perkin Elmer GeneAmp PCR 9600 System, with the parameters: denaturation at 94°C 1 minute,

annealing at 37°C for 2 minutes and extension at 72°C for 1 minute. The final extension took 10 minutes at 72°C.

Agarose gel electrophoresis. PCR amplification products were resolved on 1.5% agarose gels (Top Vision, Fermentas GmbH, St. Leon-Rot, Germany) by electrophoresis and visualized under UV light after ethidium bromide dyeing. Photos of the gels were taken with the Kodak EDAS 290 system. As a marker, a 100bp ladder (Gene-ruler, 100bp DNA ladder plus) was used. The expected amplification product was 310bp by NLV.

Sequence analyses. The nucleotide sequences of PCR-amplified fragments were determined by using ABI PRISM Model 3100 Version 3.7 in the Biological Research Centre, Hungarian Academy of Sciences, Szeged Centre of Excellence of the European Union. Sequences were identified in BLAST.

III.3. SYBR Green real-time RT-PCR (RT-PCR, DNA analyses)

The real-time PCR was performed with Light Cycler 1.5 rapid thermal cycler system (Roche Diagnostics GmbH, Mannheim, Germany).

- a) Two-step real-time RT-PCR:** The reverse transcription was performed as described above. The optimal amplification circumstances for the PCR amplification were (/sample): 6.2µl RNase-free water (Promega, Madison, USA), 0.2µl BSA, 1.0µl 25mM MgCl₂, 1.0µl Master Mix with SYBR Green (Roche Diagnostics GmbH, Mannheim, Germany), 0.3µl p289 (0.1µg/µl) and 0.3µl p290/a (0.1µg/µl). The total amount of the master mix was 9µl and 1µl cDNA was added to the master mix. The number of PCR cycles was 35, and the following temperatures were used: denaturation at 95°C for 30 seconds; in every cycle denaturation at 95°C for 0 seconds, annealing at 37°C for 20 seconds; and extension at 72°C for 15 seconds. The emission was measured at the end of the extension, when there were dsDNAs in the amplification mix. The targets were subjected to Tm analysis; the range was 65–95°C; 0.1°C/s. The melting point of the DNA was at about 85.7°C.
- b) Single-tube real-time RT-PCR method** (Eppendorf cMaster RT_{plus}PCR System). In this method, the RT/amplification mix contained the following components: 1µl RT

plus PCR buffer with 25mM Mg²⁺, 0.2μl dNTP (10mM) mix, 0.2μl BSA, 0.125μl cMaster RT Enzyme (15 U/μl), 0.1μl cMaster PCR Enzyme Mix (5U/μl), 0.1μl RNase Inhibitor (0.5μg/μl), 1μl p289 (0.1μg/μl), 0.6μl p290/a (0.1μg/μl), 1μl Master Mix with SYBR Green (Roche Diagnostics GmbH, Mannheim, Germany) and 4.675μl RNase-free water, 1μl template was used. The RT was performed at 43°C for 60 minutes and after which samples were incubated at 94°C for 2 minutes. The PCR denaturation, annealing and extension times and temperatures were: 94°C for 12 seconds, 42°C for 20 seconds, and 68°C for 20 seconds. The number of PCR cycles was 35. The emission was measured at the end of the extension, when there were dsDNAs in the amplification mix. The targets were subjected to Tm analysis; the range was 72–95°C 0.1°C/s. The melting point of the DNA was at about 85.7°C.

- c) **Two-step real-time RT-PCR method with the newly-designed primers.** The reverse transcription was performed as described above. The optimal amplification circumstances for the PCR amplification were (/sample): 2.8μl RNase-free water (Promega, Madison, USA), 0.2μl BSA, 1.0μl 25mM MgCl₂, 1.0μl Master Mix with SYBR Green (Roche Diagnostics GmbH, Mannheim, Germany), 2.0μl forward primer (10nM) and 2.0μl reverse primer (10nM). The total amount of the master mix was 9μl and 1μl cDNA was added to the master mix. The number of PCR cycles was 35, and the following temperatures were used: denaturation at 94°C for 30 seconds; in every cycle denaturation at 94°C for 0 seconds, annealing at 42°C for 20 seconds; and extension at 72°C for 15 seconds. The emission was measured at the end of the extension, when there were dsDNAs in the amplification mix. The targets were subjected to Tm analysis; the range was 65–95°C 0.1°C/s. The melting point of the DNA was at about 82°C.

III.4. Argene Calici/astrovirus Consensus kit (Argene Inc., North Massapequa, N.Y., USA)

RT-PCRs were carried out as specified by the manufacturer's instructions. The Argene Calici/Astrovirus Consensus kit, a PCR is combined with an ELISA method. The kit allows specific genome detection of all HuCVs (norovirus and sapovirus genera). After RNA isolation, reverse transcription and amplification steps, HuCVs are detected with a microtiter plate, using hybridization of their amplified product with a biotinylated probe. The kit also contains an internal control. For each amplification run, 10μl of extracted RNA was used.

III.5. Cepheid Norovirus Primer and Probe Set (Cepheid, Sunnyvale, CA, USA; real-time RT-PCR)

The Cepheid Norovirus Primer and Probe Set consist of two types of beads: norovirus beads and sample preparation control (SPC) beads. Norovirus beads contain primers and two FAM-labeled probes for the detection of GGI, and one Alexa Fluor 647-labelled probe for the detection of GGII. The norovirus beads also contain primers and a Texas Red-labeled probe for a separate sample preparation control sequence. The SPC beads contain an RNA target for sample preparation control. By using different hybridization probes, we can differentiate between the norovirus genotypes GI and GII. Working with Cepheid Norovirus Primer and Probe Set 5µl of extracted sample was used for each real-time PCR run.

III.6. IDEIATM Norovirus ELISA Test (Dakocytomation Ltd, Ely, United Kingdom)

The IDEIATM Norovirus ELISA Test is an enzyme immunoassay for the qualitative determination of noroviruses of genogroups I and II in stool samples. The IDEIATM Norovirus ELISA utilizes wells coated with GGI- and GGII-specific monoclonal antibodies. It is a very simple and rapid method.

IV. Results

IV.1. RNA isolation

The first aim of this study was to find the most effective RNA isolation method to be able to detect noroviral RNA in stool samples. Five different RNA purification methods were compared on 100 samples. The aspects considered were: the time spent for sample preparation, determination of the number of samples containing HuCV and to determine the number of aspecific products and the cost of preparation (Table 2). We got 11 positive samples when we worked with the Trizol methods and the Roche kit. By working with the V-Gene kit, we only got 8 positive samples. With the Roche kit, a significant number of PCRs were inhibited. The most rapid purification method was the Roche kit; we performed it within 40 minutes. With the V-Gene kit, the purification took 60 minutes. The longest and the most labor-intensive method were the Trizol methods.

	Trizol methods		V-Gene kits		Roche
	Trizol-Genetron	Tri-Reagent	V-Gene RNA	V-Gene Viral RNA/DNA	Roche
Time for sample preparation (minutes)	120	120	60	60	40
HuCV positive samples (N)	11	11	8	8	11
Number of aspecific products	negligible	negligible	negligible	negligible	significant
Cost of preparation (Euro/sample)	0.4	0.32	2.5	2.25	2.52

Table 2. Comparison of 5 different methods of RNA purification from 100 stool samples

We used the QIAmp Viral RNA Mini Kit for RNA purification when we applied the real-time RT-PCR kits. By using the QIAmp Viral RNA Mini kit, we could effectively eliminate PCR inhibitors. The number of inhibited PCRs was only 1 (1.61%) from 61 stool samples with the Argene Calici/Astrovirus Consensus kit and 6 (9.68%) from 61 stools with the Cepheid Norovirus Primer and Probe Set (see Table 3). Different recombinant polymerases were used in the different PCRs, the polymerases differ from one another in their sensitivity. This may explain the different number of inhibited samples.

Assay name	Total number of samples	Number of inhibited samples (%)
Argene Calici/Astrovirus Consensus kit	61	1 (1.61)
Cepheid Norovirus Primer and Probe Set	61	6 (9.68)

Table 3. Number of inhibited samples which were prepared with the QIAmp Viral RNA Mini Kit

IV.2. Detection of noroviral RNA by using real-time RT-PCR technique

The traditional RT-PCR technique is a very reliable but time-consuming method. If there is a suspicion of norovirus infection in hospital wards, it is essential to examine samples and publish result as soon as possible. We decided to introduce widely used real-time PCR for the norovirus diagnosis (Figures 6 and 7).

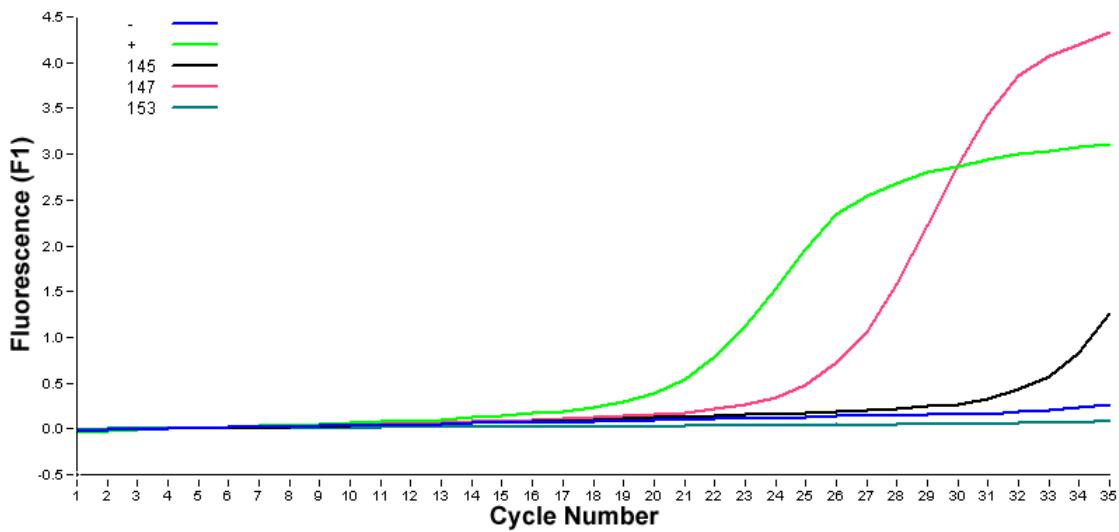


Figure 6. The chart depicts negative, positive controls and 3 samples. There amplification accured in capillaries containing positive control, samples number 145 and number 147

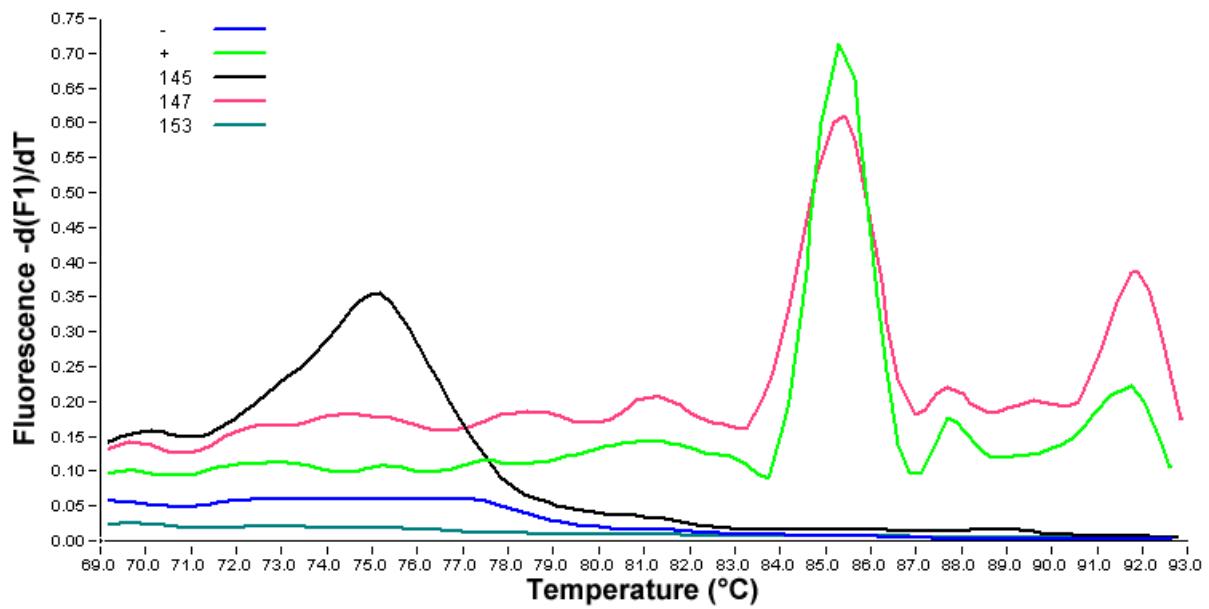


Figure 7. The chart depicts negative, positive controls and 3 samples. Two samples were negative (numbers 145 and 147), number 145 contained primer dimers. The melting point of the dimers was 10°C below the melting point of the norovirus-containing sample (147), which was at about 85.7°C

For the confirmation of the real-time PCR assays, an agarose gel-electrophoresis was used. An agarose gel-electrophoresis run is seen in the next figure (Figure 8).

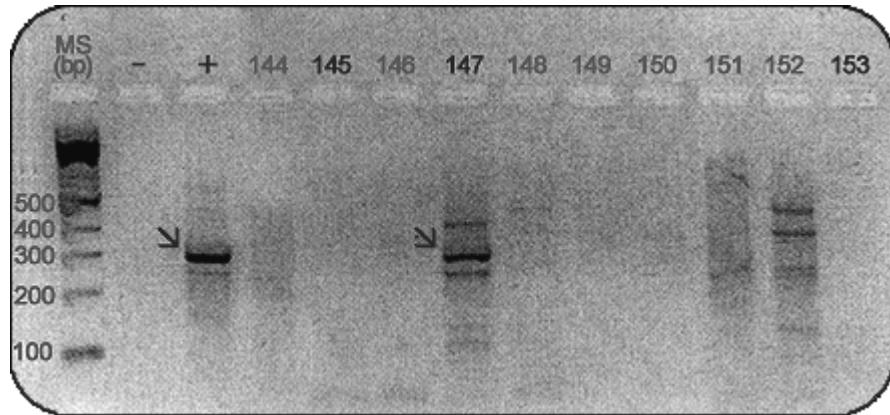


Figure 8. The gel-electrophoretic result of the amplified samples. In the first well, the molecular-weight marker is seen (Gene-ruler, 100bp DNA ladder plus), followed by the negative and the positive control and the clinical samples (144–153). The 310bp product is seen in the positive control well and in sample number 147. Aspecific products are also displayed among controls and samples

After each run, some of the norovirus containing samples were sequenced.

Sample number 76:

5'-

CATGGATGGAAATCCTTCAACATTATGTGTAAGCTCACAGCCGACCCCTCCTGG
 CCGTAGTGGTGGCACAGGATTACTCTCCCCATCTGAAATGGACGTTGGCGACTA
 TGTGAAACAGTGTCAAAGATGGTCTGCCATCTGGCTTCCATGCACTCAAAGGTG
 AATAGCATTAAACCACTGGATCCTAACCCATGTGCACTGTCAGAAGTCACTGGCT
 TGTCCCCAGATGTGATACAATCACGATCCTACTTCTCATTCTACGGTGAG-3'

The nucleotide sequence was inserted to the BLAST and the result was the following:

Nucleotide sequence accession number: AJ487811.1| Human calicivirus
 NLV/Benetusser/453/2002/Sp partial pol gene for RNA-directed RNA polymerase, genomic
 RNA Length=307 bp, Identities 97%.

30 samples were compared by using the traditional method and the two-step real-time PCR method (by using the Jiang-designed primers). With both methods, there were 4 negative and 3 positive samples. 3 samples were positive with traditional PCR and negative with two-step real-time PCR. 20 samples were positive with real-time PCR and negative with traditional PCR (Table 4).

	Two-step real-time PCR		Σ
	Positive samples	Negative samples	
	N (%)		
Traditional PCR positive samples	3 (10)	3 (10)	6
Traditional PCR negative samples	20 (66.7)	4 (13.3)	24
Σ	23	7	30

Table 4. Comparison of 30 samples by using traditional and two-step real-time PCR methods

On the basis of the result of our comparative study, between November 1, 2003 and January 31, 2005, 522 stool samples were analyzed with the real-time RT-PCR assay. On using the two-step rt-PCR by the Jiang-designed primers, we found that 38 (9.92%) of the 383 samples contained HuCV. Of the remaining 139 samples, 14 (10.07%) proved positive for HuCV on the use of the one-step rt-PCR method by the Jiang-designed primers.

IV.3. Results with the new primer-pairs

A mathematical algorithm was designed by using all the norovirus sequences were found on the BLAST database. The sequences were sorted according to the number of norovirus sequences they occur. The most specific forward primer was chosen, and then a reverse primer, the melting point of which was close to the forward primer's melting point.

We compared both primers on 110 samples by using traditional RT-PCR. We found that 32 (29.1%) samples were positive by using our newly designed primers, but negative with the Jiang-designed ones. There was only one (0.9%) sample, which was positive with the Jiang-designed primers, but negative with the new primers. 5 samples were positive and 72 samples were negative with both methods (Table 5).

Jiang-designed primers p289 and p290a	Positive		Negative
			(%)
New primers			
Positive	5 (4.5)		32 (29.1)
Negative	1 (0.9)		72 (65.5)

Table 5. Comparison of 110 samples using the Jiang-designed (p289 and p290/a) and the newly designed primers with the traditional RT-PCR method

The real-time PCR was optimized (Figures 9 and 10), and the confirmation of the PCR products (130bp) were analyzed by gel-electrophoresis (Figure 11) and by sequencing.

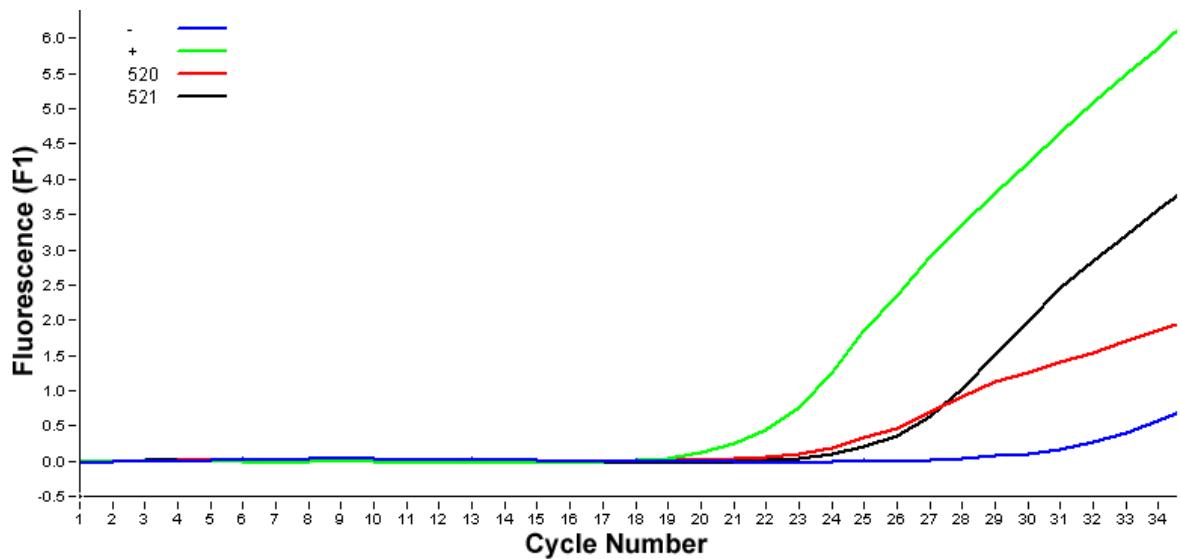


Figure 9. The chart depicts negative, positive controls and 2 samples, 520 and 521. The PCR amplification curves are seen. There was amplification in all capillaries

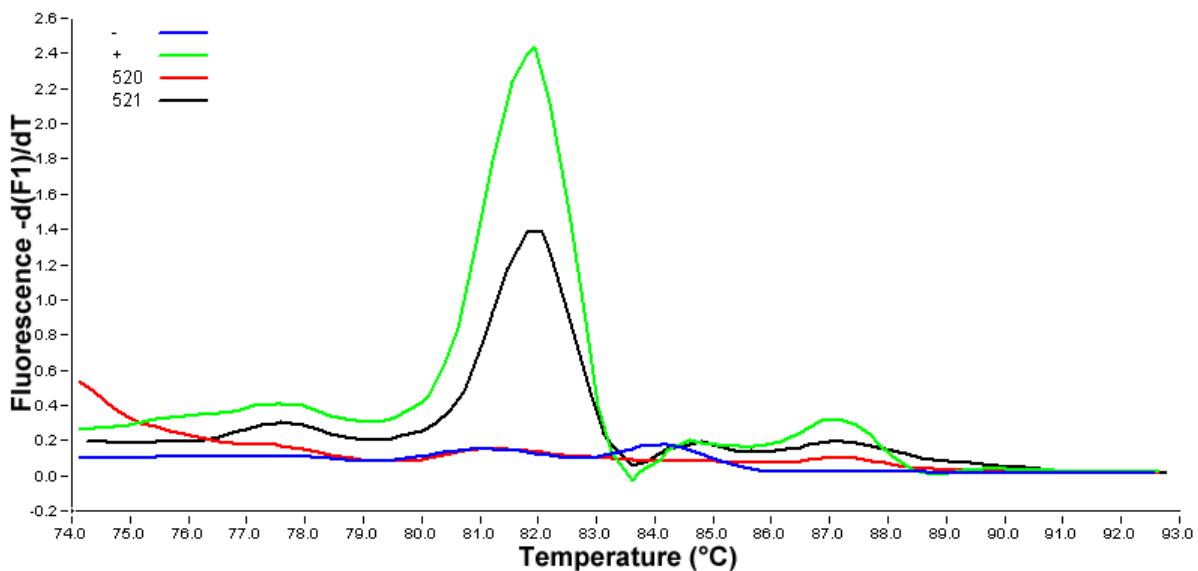


Figure 10. The chart depicts negative, positive controls and 2 samples. One sample was negative (number 520). The melting point of the norovirus-containing sample (521) was at $82.0 \pm 0.5^{\circ}\text{C}$

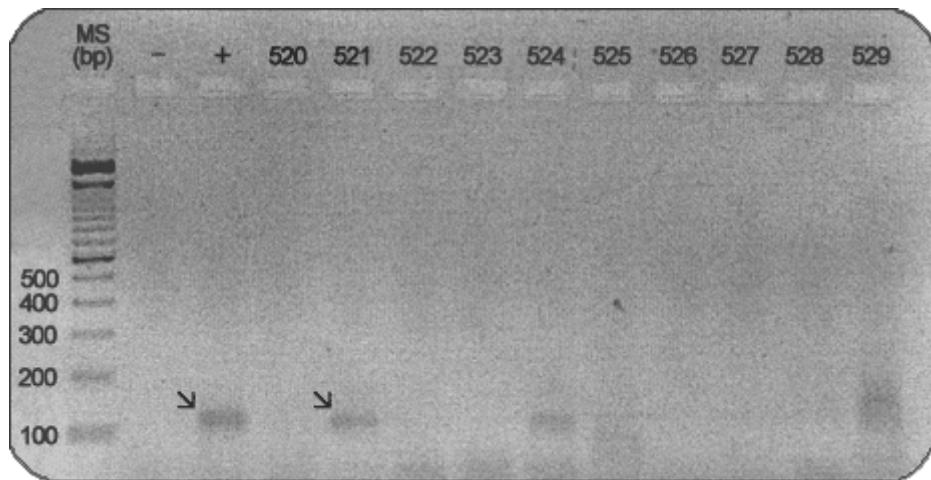


Figure 11. The gel-electrophoretic result of the amplified samples. In the first well, the molecular-weight marker is seen (Gene-ruler, 100bp DNA ladder plus), followed by the negative and the positive control then samples (520–529). The 130bp product is seen in the positive control well and in sample number 521. It is seen that there are not formed aspecific product during the RT-PCR

66 samples were compared by the two (using the Jiang-designed and our newly-designed primers) two-step real-time RT-PCR method. 12 (18.1%) proved to be positive with the newly-designed primers and only 4 (6.06%) were positive with the Jiang-designed ones.

We further analyzed 514 samples by real-time RT-PCR with the Jiang-designed primers. 56 (10.9%) samples proved to be positive with these primers. After optimization, the real-time

RT-PCR with the new primers, we made the norovirus diagnosis with this new primer pair. We examined 183 samples with this primer-pair and found 41 (22.4%) norovirus positive stool.

IV.4. ELISA technique compared to two commercial norovirus RT-PCR kits

RT-PCR is an expensive method, and its use requires special equipment, devices and skills. In the routine laboratory, a faster and simpler method is needed. At present, many norovirus antigen detection ELISA systems are commercial available. The IDEIA™ Norovirus ELISA kit was used, as in earlier studies it was shown to have the highest sensitivity compared to other ELISA systems^(37,38).

In this comparative study 61 stool samples were examined for noroviruses. The Kappa coefficient was calculated to determine the level of agreement between assays (IDEIA™ Norovirus ELISA vs. Argene Calici/Astrovirus Consensus kit, IDEIA™ Norovirus ELISA vs. Cepheid Norovirus Primer and Probe Set, Cepheid Norovirus Primer and Probe Set vs. Argene Calici/Astrovirus Consensus kit); sensitivity, specificity, positive (PPV) and negative (NPV) predictive values were determined. A patient was adjudged to be infected with norovirus if the stool sample proved positive with one of the two RT-PCR methods, because the RT-PCR offers the ability to detect lower levels of the virus comparing to ELISA systems. The age of the patients involved ranged between 4 days and 83 years (median age: 7.5 years, mean age: 28.0 years). The male/female ratio was 25/36. There were no cases of repeated infections during the study period. Of the 61 samples, 52 were positive for norovirus, 47 of the 52 positive being true-positive. The results are presented in Table 6, 7 and 8 and Figure 12.

Methods	Positive	Negative	Borderline	Inhibitors
	N (%)			
Argene Calici/Astrovirus Consensus kit*	48 (79.1)	10 (16.1)	2 (3.2)	1 (1.6)
Cepheid Norovirus Primer and Probe Set	47 (77.4)	8 (12.9)	0 (0)	6 (9.7)
IDEIA™ Norovirus EIA	38 (61.3)	23 (38.7)	0 (0)	0 (0)

Table 6. Comparison of three methods for the diagnosis of norovirus in diarrheal fecal samples (N=61)

The IDEIA™ Norovirus ELISA revealed 38 norovirus-positive and 23 negative samples. The Kappa coefficient between the ELISA and the Argene Calici/Astrovirus Consensus kit was 0.50 (standard error (SE): 0.12; 95% confidence interval (CI): 25%–75%), the Kappa coefficient between the ELISA and the Cepheid Norovirus Primer and Probe set was 0.42 (SE: 0.14; 95% CI: 14%–69 %). The sensitivity of the test was 78.9%, the specificity was 100%, the PPV was 100% and the NPV was 39.1%.

By using the Argene Calici/Astrovirus Consensus kit of the 61 samples, 48 were positive for HuCVs, 10 were negative, 2 were borderline and 1 contained inhibitors. The sensitivity of the test was 92.8%, the specificity was 100%, the PPV was 100% and the NPV was 69.2%. The Kappa coefficient between the Argene Calici/Astrovirus Consensus kit and the Cepheid Norovirus Primer and Probe set was 0.55 (SE: 0.14; 95% CI: 27%–82%).

With the Cepheid Norovirus Primer and Probe Set, 47 of the 61 stool samples proved to be positive for human noroviruses; 46 were GGII and only 1 was GGI-positive. 8 samples were negative and only 6 contained inhibitors. The sensitivity of the test was 91.2%, the specificity was 100%, the PPV was 100% and the NPV was 64.3%.

	Kappa coefficient	Standard error (SE)	95 % Confidence interval (CI %)
IDEIA™ ELISA vs. Argene Calici/Astrovirus Kit	0.50	0.12	25–75
IDEIA™ ELISA vs. Cepheid Norovirus Primer and Probe Set	0.42	0.14	14–69
Argene Calici/Astrovirus Kit vs. Norovirus Primer and Probe Set	0.55	0.14	27–82

Table 7. Statistical analyses of the methods used for the diagnosis of norovirus in diarrheal fecal samples

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
IDEIA™ ELISA	78.9	100	100	39.1
Argene Calici/Astrovirus Kit	92.8	100	100	69.2
Cepheid Norovirus Primer and Probe Set	91.2	100	100	64.3

Table 8. Sensitivities, specificities, PPV and NPV values of the methods used for the diagnosis of norovirus in diarrheal fecal samples

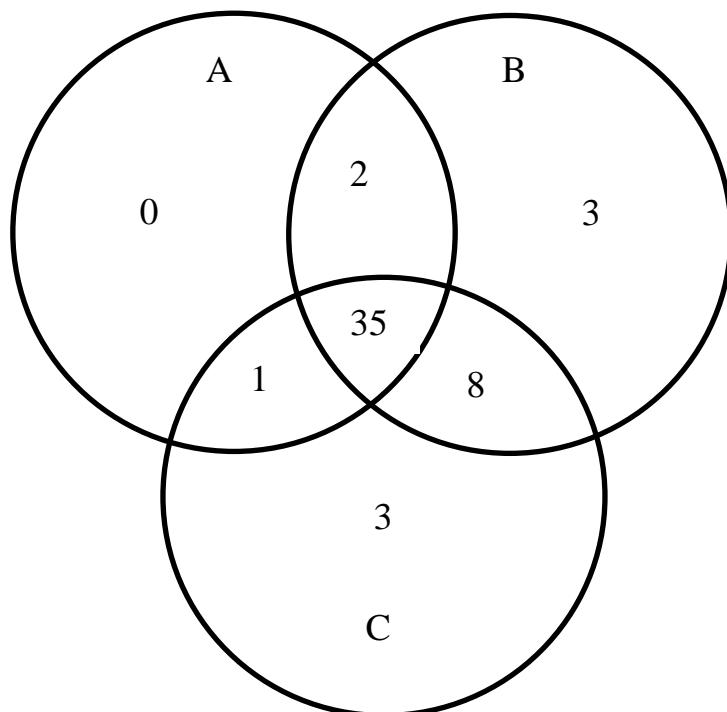


Figure 12. **A:** Number of positive samples with the IDEIA Norovirus ELISA. **B:** Number of positive samples with the Argene Calici/astrovirus Consensus kit. **C:** Number of positive samples with the Cepheid Norovirus Primer and Probe Set

IV.5. The pathogenic role of noroviruses in sporadic cases and in epidemics

Between 1 January 2004 and 31 March 2007, 1,152 stool samples were collected from children in the age group between 0 and 3 years.

Samples were sent from the Pediatric Isolation Ward (182), the In-patient Pediatric Ward (636) and the Outpatient Pediatric Ward (146) of the University of Szeged and from the pediatric patients of family doctors (188).

Conventional RT-PCR was performed with the primer pairs designed by Jiang, p289 and p290/a. Real-time RT-PCR with the primer pairs designed by Jiang and/or with our own, newly-designed primer pair; and the Norovirus I/II antigen-ELISA kit (DakoCytomation), Norovirus I and II antigen-ELISA kit (IDEIATM) methods were used for norovirus diagnosis. Biostatistical software (SPSS15 for Windows) was applied to compare mean ages and sex distributions in the two groups of positive and negative test results (two-sample t-test and χ^2 -test).

Of the overall 1,152 stool samples, 186 (16.2%) proved positive for noroviruses (Table 9). Of the 182 stool samples from the Pediatric Isolation Ward, 53 (29.1%) were positive, of the 636 stool samples from the In-patient Pediatric Ward, 87 (13.7%) were positive, of the 146 stool samples from the Outpatient Pediatric Ward 22 (15.1%) were positive, and of the 188 stool samples from the family doctors, 25 (13.3%) were positive.

The mean value (SD) in the case of negative samples was 1.179 (0.9704) and in the case of positive samples was 1.266 (0.9813); the difference was not significant ($t=-1.114$, $df=1150$, $p=0.266$). 18.06% of the 620 males and of the 532 females 14.09% were positive. Samples were from 620 males and 532 females patients. 112 male samples and 75 female samples proved positive. The difference was not significant ($\chi^2=3.313$, $df=1$, $p=0.069$).

Age (y)	Negative samples	Positive samples	Total
	N (%)		N
0-≤1	516 (84.8)	92 (15.2)	608
1-≤2	239 (83.5)	47 (16.5)	286
2-≤3	211 (81.8)	47 (18.2)	258
Total	966	186	1,152

Table 9. The incidence of human norovirus gastroenteritis in 3 age groups

Between 1st January, 2003 and 16th June 2011, 5,031 samples were examined for noroviruses, from which 836 (16.6%) proved to be positive. The following table (see Table 10) demonstrates the annual distribution of noroviruses, rotaviruses, enteric adenoviruses and human astroviruses. Although twice as many stool samples were sent for rotavirus antigen detection, the average of positive samples remained below that of noroviruses (rotavirus positives samples: 12.1%, norovirus positive samples 16.6%).

Year	Nº of samples / rotavirus positives (%)	Nº of samples / adenovirus positives (%)	Nº of samples / astrovirus positives (%)	Nº of samples / norovirus positives (%)
2003	229/17 (7.4)	229/6 (2.6)	0	37/9 (24.3)
2004	497/24 (4.8)	496/38 (7.7)	0	115/23 (20)
2005	889/36 (4.1)	888/23 (2.6)	0	492/70 (14.2)
2006	2,067/262 (12.7)	2,096/89 (4.3)	150/17 (11.3)	537/100 (18.6)
2007	1,525/169 (11.1)	1,525/44 (2.9)	84/1 (1.2)	883/289 (32.7)
2008	2,309/459 (19.88)	2,309/66 (2.9)	266/6 (2.3)	686/82 (11.9)
2009	2,334/282 (12.1)	2,334/55 (2.4)	137/2 (1.5)	795/70 (8.8)
2010	2,378/198 (8.33)	2,376/96 (4.0)	0	963/106 (11.0)
2011	1,255/189 (15.1)	1,250/37 (2.9)	0	523/87 (16.6)
Σ	13,483/1,636 (12.1)	13,503/454 (3.4)	637/26 (4.1)	5,031/836 (16.6)

Table 10. The number of rotavirus, adenovirus, astrovirus and norovirus positive samples between 2003 and 2011

Table 11 shows the total number of norovirus tests per year. It is seen that the number of test carried out increased year by year. The average rate of positivity was 16.6%.

We decided to analyze samples not only from epidemics, but also from sporadic cases. We compared the number of test which were carried out from in-patient children stool samples (age below 14 years) and from out-patient children samples. The norovirus positivity rate was higher in in-patient samples than in samples from out-patients (results are shown in Table 12).

	Total number of tests			Total number of positive samples		
	♀	♂	Σ	♀	♂	Σ (%)
2003	22	15	37	2	7	9 (24.3)
2004	49	66	115	12	11	23 (20)
2005	258	234	492	36	34	70 (14.2)
2006	256	281	537	35	65	100 (18.6)
2007	450	433	883	155	134	289 (32.7)
2008	353	333	686	43	39	82 (11.9)
2009	408	387	795	36	34	70 (8.8)
2010	541	421	963	63	43	106 (11)
2011	259	264	523	40	47	87 (16.6)
Σ	2,596	2,434	5,031	422	414	836 (16.6)

Table 11. The distribution by gender of samples sent to the lab for norovirus diagnosis between 2003 and 2011.

	Total number of samples from children	Number of samples from outpatient children	Number of positive samples from outpatient children (%)	Number of samples from in-patient children	Number of positive samples from in-patient children (%)
2003	12	4	0 (0)	8	2 (25)
2004	90	65	7 (10.7)	25	13 (52)
2005	400	180	28 (15.5)	220	32 (14.5)
2006	464	112	23 (20.5)	352	64 (18.2)
2007	633	70	13 (18.6)	563	186 (33)
2008	489	248	25 (10.1)	241	31 (12.9)
2009	500	320	22 (6.9)	180	12 (6.7)
2010	535	268	16 (5.9)	267	32 (11.9)
2011	246	83	4 (4.8)	163	25 (15.3)
Σ	3,369	1,350	138 (10.2)	1,799	397 (22.1)

Table 12. Number of positive samples arrived from children (age <14 years) for norovirus diagnosis

In Hungary, 50–100 calicivirus epidemics acquired in different communities have been registered between 2004 and 2009³⁹. During our 9-year study, we only found 10 accumulations in hospital units. (Table 13).

	Total number of norovirus accumulations	Location of norovirus accumulations
2003	0	-
2004	2	Chronic Ward of the Pediatric Department (4 people) General Ward of the Pediatric Department (4 people)
2005	0	-
2006	1	First Department of Internal Medicine (4 people)
2007	2	First Department of Internal Medicine (Hematology, 6 people) First Department of Internal Medicine (General Ward, 8 people)
2008	1	Chronic Neurology (5 people)
2009	0	-
2010	1	First Department of Internal Medicine (Gastro-Hepatology, 4 people)
2011	3	Psychiatry (5 people) First Department of Internal Medicine (Gastro-Hepatology, 13 people) Second Department of Internal Medicine (Cardiology, 5 people)
Σ	10	

Table 13. Total number of norovirus accumulations at the different units of the Albert Szent-Györgyi Clinical Center between 2003 and 2011

V. Discussion

V.1. RNA isolation

Methods based on PCR have replaced many traditional virus detection assays in clinical virus laboratories. In spite of their superior sensitivity, they have also weaknesses which limit their use in virus diagnostics. One of these problems is caused by PCR inhibitors which are often present in clinical samples and may lead to false negative results⁴⁰. Such inhibition can be detected by using an internal control in the PCR reaction. The quality and quantity of inhibitors vary between samples, and several kinds of PCR inhibitors have been characterized including phenolic compounds, glycogen, fats, cellulose, non-target nucleic acids and heavy metals⁴¹. Various methods have been developed to remove or inactivate these inhibitors. Many nucleic acid extraction methods can eliminate some parts of the PCR inhibitors, but their efficiency varies and is far from complete. Previous studies have shown that inhibitors can partly be inactivated or bound by several compounds such as betadine, bovine serum albumin, formamide, glycerol and tween⁴². The amount of PCR inhibitors can also be reduced after nucleic acid extraction, but these methods are time-consuming and reduce the yield of nucleic acids, limiting their value in clinical diagnostics.

We compared all RNA extraction methods, two Trizol-based methods, and three kits for viral RNA isolation for preparing 100 fecal samples.

Using Trizol methods, the efficacy was 11%; the number of aspecific products was negligible and are cheap methods. However, experienced staff is necessary to carry out this procedure, and it takes two-times longer to get purified RNA than with the kits. By the Trizol RNA isolation methods, inhibitors have been eliminated very effectively.

By working with the V-Gene kits, the efficacy was 8%, the number of aspecific products was negligible. The time for sample preparation took 60 minutes and the price was 6 times more compared to Trizol-methods. By using these RNA isolation methods, inhibitors have been eliminated very effectively, but we lost RNA in three cases.

The Roche kit provides a special buffer for removing the PCR inhibitors. In spite of this, we got most inhibited samples by working with this kit. Although it is a time-saving and labor friendly method, the efficacy was high (11%), but it was the most expensive one. When we prepared nucleic acid from samples such as blood or respiratory samples, much fewer inhibitory samples were detected compared to RNA isolation from stool samples.

Our study showed that the most effective method would be the traditional, Trizol-based RNA purification method. In our laboratory, many PCR-based diagnostic methods are available, so we had to choose a time-saving and effective nucleic-acid isolation method. We decided to use the V-Gene RNA/DNA Preparation kit.

There are many RT-PCR kits commercially available for the detection of noroviruses. These kits recommend to the user which way the RNA should be performed. Both Argene and Cepheid kits recommend the use of the Qiagen Viral RNA Mini kit. The QIAamp Viral RNA Mini Kit simplifies isolation of viral RNA with fast spin-column or vacuum procedures. No phenol-chloroform extraction is required. Viral RNA binds specifically to the QIAamp silica membrane while contaminants pass through. PCR inhibitors such as divalent cations and proteins are completely removed in two efficient wash steps, leaving pure viral RNA to be eluted in either water or a buffer provided with the kit. The time for sample preparation was 20-40 minutes. By using the QIAamp Viral RNA Mini kit, we effectively eliminated PCR inhibitors from the examined 61 samples. The number of inhibited PCRs was only 1 (1.61%) with the Argene Calici/astrovirus Consensus kit, and 6 (9.68%) with the Cepheid Norovirus Primer and Probe Set. Different recombinant polymerases are incorporated in the different PCR kits, the polymerases differ from one another in their sensitivity. This may explain the different number of inhibited samples.

Oikarinen et al. showed that PCR inhibitors are relatively common in stool samples and their effect can strongly influence the results of PCR assays. In altogether 19% of the stool samples the amount of PCR inhibitors was so high that they led to false negative results when using PCR-based assays for virus diagnosis. They also found that the frequency of PCR inhibitors was even higher in the adult population compared to that observed in the young infants. In their study, no inhibition was found in infants younger than 6 months old. Oikarinen et al. tested the effect of BSA added to both RT and PCR reactions as a factor eliminating the effect of inhibitors. BSA treatment decreased the efficacy of PCR amplification in samples which did not contain any PCR inhibitors. However, all stool samples were positive when BSA was added to the RT and PCR reactions. This indicates that the addition of BSA reduced the sensitivity of the RT-PCR method, but at the same time, BSA effectively inactivated the RT-PCR inhibitors⁴¹.

V.2. Efficiency of norovirus real-time RT-PCR technique

Since the cloning of the Norwalk virus in 1990, RT-PCR assays have been developed for the detection of noroviruses in clinical and environmental specimens, such as water and food⁴³.

RT-PCR assays are used widely in commercial and research laboratories allowing for the detection of virus in specimens collected late in illness, when the quantity of the virus is low. RT-PCR followed by nucleotide-sequencing has been particularly useful in molecular epidemiology studies to identify point-source of infection, as well as to differentiate outbreaks that were mistakenly assumed to be connected⁴⁴. Real-time quantitative RT-PCR, which is faster and more sensitive than conventional RT-PCR, has been developed for rapid detection of noroviruses in stool samples. The first step was to perform the PCR on a real-time instrument. We used the Light Cycler 1.5 (Roche) instrument, which has 3 optical channels. SYBR Green was used in our PCR to detect dsDNAs in the PCR mixture. SYBR Green I bind to dsDNAs. The resulting DNA-dye-complex absorbs blue light ($\lambda_{\text{max}} = 488\text{nm}$) and emits green light ($\lambda_{\text{max}} = 522\text{nm}$). SYBR Green I is marketed as a replacement for the mutagen ethidium bromide, as it is both safer to work with and free from the complex waste disposal issues of ethidium. However anything capable of binding DNA with high affinity is a possible carcinogen, including SYBR. Another advantage of the SYBR Green is that we can obtain a melting curve data. The melting curve is beneficial in verifying the presence of authentic amplicons as well as primer dimer or spurious product.

We compared the traditional RT-PCR and the two-step real-time PCR methods. There were 20 samples which were positive with the SYBR Green real-time PCR method, but negative with the traditional PCR. Real-time PCRs are more sensitive than traditional PCRs, thanks to the dyes and hybridization probes. Another advantage is that in real-time PCRs we do not need agarose gel-electrophoresis, because the detection of the amplified product happens in real-time. 3 samples were positive with traditional RT-PCR, but negative with real-time PCR; this can be due to the fact that these samples were inhibited in the SYBR Green assay.

Pang et al. collected their samples from sporadic cases and from epidemics. All of the samples which proved to be positive with traditional PCR were positive with the Light Cycler real-time PCR method. They established the detectable RNA amount, as $5-5 \times 10^6$ copies RNA/reaction, which means 25,000 copies RNA/g stool⁴⁵.

Our data clearly demonstrate that the real-time PCR systems are excellent diagnostic tools, because they are more specific, more sensitive and faster than traditional PCR methods. The only one disadvantage is that we did not need an internal control during real-time PCR assays. The next step was to adapt the complete assay (reverse transcription and PCR) to the Light Cycler 1.5 instrument. With this method, the time for reverse transcription has been reduced, the one step protocol simplifies the method and reduces the risk of contamination of the RNA; moreover, it is useful for routine diagnosis as there is no post-amplification processing of the product. Pang et al. found in 2005 that a single-step protocol for noroviruses has resulted in

reduced sensitivity compared to the two-step RT-PCR⁴⁶. Working with the one-step real-time RT-PCR assay, we got as many positive results (10.07%) as with the two-step real-time PCR (9.92%).

Richards et al. also described a SYBR Green, real-time RT-PCR method to detect a commonly used research strain of noroviruses. This method was specific for the 8FIIa Norwalk virus cluster and for the Matsui-designed primer pair (specific for GGI noroviruses). They also found that the real-time RT-PCR technique using SYBR Green fluorescence followed by melting temperature determination was simple and effective method in identifying the Norwalk virus in stool, and the use of SYBR Green allowed for an initial product verification by simple examination of first derivative melt graphs⁴⁷.

Scipioni et al.⁴⁸ developed a sensitive and broadly reactive TaqMan real-time RT-PCR assay which has the unique features of detection and quantification of human GGI and GGII and bovine GGIII noroviruses with the same set of primers and probe. Other advantages of this assay are the ability to detect inhibitors of PCR or RT-PCR that may be present in stool and to estimate the viral load of norovirus in the sample. The method uses a one-step hot start, RT-PCR with thermo stable DNA polymerase. Scipioni et al. quantified the viral load. The majority of positive human samples contained from 10^7 to 10^{10} copies of norovirus/gram of stool sample (this fact explains the high infectivity of the virus: lots of viruses are in stool and 10–100 virus particles are enough to infect). This value is much higher than those previously estimated by EM⁵.

In the past years many researchers have tried to develop a multiplex real-time system with which not only noroviruses but also adenoviruses, astroviruses, rotaviruses and sapoviruses are detectable. Of course, a big advantage of such an assay is the possibility to detect viruses only in one tube by PCR. The disadvantage of the multiplex assay is the fact that sensitivity decreases.

Maarseveen et al. developed a multiplex real-time assay, which contained two internal controls. By using this assay, it is possible to detect simultaneously Astrovirus, Adenovirus group F, Rotavirus, Norovirus GGI and GGII and Sapovirus. They found higher C_T values in multiplex real-time PCR compared to the monoplex assays and the resulting loss in sensitivity, for adenoviruses were not considered a major disadvantage. "Since fewer than 5% of the samples contained multiple infections in practice, the observed loss of sensitivity for norovirus GGII and sapovirus upon co-amplification was accepted"⁴⁹.

99% of the positive samples belonged in GGII and only 1 in GGI. This is consistent with the findings of other studies of sporadic cases of gastroenteritis in children^{50,51,52,53}. Norovirus GGI strains constitute a minor proportion of strains in most outbreak

studies^{51,54,55,56}. Maslin et al. found that among noroviruses, GII were predominant as it was previously shown in industrialized countries⁵⁷. Our results show the same result: predominantly GGII plays an important role in both sporadic cases and outbreaks of acute gastroenteritis, not only in infants and young children, but also in adults.

Stelten et al. designed a new assay for the improved detection of GGI norovirus in patient samples. Their redesigned assay demonstrated a 64-fold increase in sensitivity, a 2-log decrease in the limit of detection, and an 18% increase in amplification efficiency, when compared to the standard assay. The optimized test also detected GGI norovirus in clinical specimens that were initially negative by the standard assay. Use of the optimized assay increased the annual positivity of GGI norovirus from 1.2% to 4.5%, indicating that the prevalence of GGI norovirus may be higher than previously identified⁵⁸.

V.3. Application of the new primer-pairs

While the available primers for RT-PCR assays detect many strains of noroviruses, some strains may escape detection. The Jiang-designed primer pair is able to detect not only noroviruses but also sapoviruses. While working with this primer set, we discovered that many aspecific products formed during the PCR. We decided to generate a new primer pair with which norovirus diagnostic would become more specific.

66 samples were compared by the two two-step real-time RT-PCR method. 12 (18.1%) proved to be positive with the newly-designed primers and only 4 (6.06%) were positive with the Jiang-designed ones. Our result shows that our primers are more specific than the Jiang-designed ones⁵⁹.

It is seen that norovirus primers have to be chosen according to geographical location (different norovirus strains circulating in the US and in Europe) and to the goal of the research (diagnosis or epidemiological study) is to find appropriate primer with which most of the noroviruses become detectable.

We have developed a primer pair (targeting the RNA-depending RNA polymerase region) with which norovirus diagnostics become in the European region safely practicable.

V.4. Efficacy of the ELISA technique compared to two commercial norovirus RT-PCR kits

Various commercial stool EIA detection methods have been developed. EIA assays are highly specific for some noroviruses, but, in general, not sensitive enough to detect a wide range of

noroviruses. Newer generation EIAs based on antibodies against a wider range of baculovirus-expressed viral antigens are being developed and tested, however, the sensitivity of EIAs still remains limited⁶⁰. The high specificity of these assays make them useful for diagnosing noroviruses in outbreak investigations where many specimens may be available and confident detection of virus in few cases might be sufficient for etiologic confirmation. The sensitivity of these assays is genotype-dependent and results can vary based on the diversity of circulating strains in the population⁶⁰. However, in clinical practice, it is important to obtain results as soon as possible in order to minimize the spread of the virus within and between hospital units.

We found 14 RT-PCR positive samples which were negative by the ELISA. This can be due to the fact that the detection of norovirus in stool samples by the ELISA is based on the detection of viral antigens and may thus be hampered by the antigenic diversity of noroviruses. 3 samples were positive by using the Cepheid kit but negative with the Argene kit. This can be explained by the fact that the Cepheid RT-PCR is a real-time PCR, with fluorescent probes and its sensitivity is much higher than that of commercial PCR-s. 3 samples were positive with the Argene kit and negative with the Cepheid kit and ELISA. We believe that these 3 samples contained sapporoviral RNA. The ELISA kit was more specific and sensitive, as de Bruin et al. previously described^{60,61}. They found that the sensitivity of ELISA was only 38% and the specificity 96%.

The RT-PCR assay is a powerful alternative method for the laboratory diagnosis of non-bacterial, acute gastroenteritis; however, due to the high genetic diversity, constant updating of primers (and probes) is required which adds to the complexity of RT-PCR as a diagnostic method. Genome amplification also allows the molecular characterization of viral strains.

V.5. Significance of the pathogenic role of noroviruses in sporadic cases and in epidemics

With RT-PCR assays as a diagnostic tool, it has been established that human noroviruses are the most common causes of gastroenteritis outbreaks worldwide^{62,63,64}. There have been only a few reports on the presence of noroviruses in sporadic cases^{66,67}. Frogatt et al. found that during the winter season noroviruses were the second most common agents of acute non-bacterial gastroenteritis⁶⁶.

Noroviruses are listed as the most common causes of non-bacterial gastroenteritis (ahead of Rotavirus) worldwide. The detection of Noroviruses in clinical samples has been hampered

with the inability to propagate the virus in the laboratory and the complexity of virus extraction and assay by molecular methods. In the past years we have found the best nucleic acid extraction method, introduced the traditional RT-PCR, translated to real-time RT-PCR, designed new primers and introduced an ELISA with which a high number of samples are investigated simultaneously. It is important to reveal the importance of noroviruses not only in epidemics but also in sporadic cases in different health-care units.

Between 2004 and 2007 we examined the presence of noroviruses in sporadic gastroenteritis cases and found that in children in the age group between 0 and 3 years, noroviruses are common causative agents of non-bacterial gastroenteritis. We also examined the presence of noroviruses in the different pediatric wards. Most positive cases come from the Pediatric Isolation Ward (29.1%), and lower numbers of positive samples from the family doctors (13.3%) and from the In-patient (13.7%) and Outpatient (15.1%) Pediatric Wards⁶⁵.

Rotaviruses are the most common causes of acute, non-bacterial gastroenteritis worldwide. The clinical symptoms of rotavirus gastroenteritis cannot be differentiated from those of the other forms of viral gastroenteritis. The consequences of rotavirus infection include home-nursing (1:1), doctor-visiting (1:5), hospitalization (1:65) and death (1:293). Gastroenteritis in young children is very often caused by astrovirus. Mustafa et al. found that 3% of the cases of children hospitalized because of gastroenteritis were due to astrovirus. 65.2% of gastroenteritis was caused by rotaviruses, 6.3% were bacterial infections, and adenoviruses were the causative agents in 4.1%⁶⁸.

Several studies have found that HuCVs are second only to rotaviruses as the cause of viral gastroenteritis in young children^{66,69}. Buesa et al. studied the importance of HuCVs in sporadic cases and in outbreaks in children under 5 years of age. In sporadic cases, HuCVs were detected in 14.19%. Rotaviruses were the most frequent agents of gastroenteritis (25.3%), and astroviruses and human adenoviruses each accounted for 3.15%. The majority of HuCV outbreaks occurred either in schools (42.8%) or in nursing homes (21.4%). The rate of noroviruses as the etiologic agents of gastroenteritis outbreaks was 56%⁷⁰. Maslin et al. investigated adults and found that the main agent of gastroenteritis were human caliciviruses (25.3%) followed by adenoviruses and astroviruses (4.8%)⁵⁷. We found that noroviruses likewise play an important role in sporadic gastroenteritis cases in Hungary. 16.2% of the stool samples contained norovirus. Most positive samples came from the Pediatric Isolation Ward. As a cause of viral gastroenteritis, we found rotaviruses in second place (10.5%), followed by human adenoviruses (4.6%). We did not find any sapoviruses between 2004 and

2007. Sapovirus-associated outbreak in Hungary was first described in 2009 by Pankovics et al⁷¹. Some reviews examined the role of noroviruses as a cause of severe gastroenteritis among hospitalized patients. Noroviruses were accounted for approximately 12% of severe gastroenteritis cases among children <5 years of age worldwide^{72,73,74,75,76}.

We showed that between 2003 and 2011 noroviruses caused the most cases of sporadic gastroenteritis (16.6%) followed by rotaviruses (12.1%). The gender distribution was 49.6%:50.4% (male: female). We found more norovirus positive cases in the in-patient samples than in the out-patient one. This can be explained by the fact that many children were admitted to hospital and examined for all enteric viruses, rota-, adeno- and noroviruses. In many cases, GPs do not send samples for norovirus diagnosis; they send stools only for rotavirus and adenovirus examinations.

We applied biostatistical methods to examine possible correlations between age or sex and positive results. At the 95% confidence interval, we did not find a significant difference between these categories, which means that in the age group of children between 0 and 3 years, there is no age or sex preference.

Noroviruses are the most common causes of gastroenteritis in all age groups, accounting for greater than 90% of viral gastroenteritis and ~50% of all-cause outbreaks worldwide^{77,78,79,80}. Foods implicated in outbreaks of norovirus gastroenteritis are contaminated either directly with fecal matter at the source (e.g., shellfish harvested from sewage contaminated water or raspberries irrigated with sewage) or by infected food-handlers⁸¹. Shellfish concentrate noroviruses through filtration. However, the most common food vehicle for noroviruses remains ready-to-eat foods, such as sandwiches and salads, particularly those that require handling but no subsequent cooking⁷⁷. Because of low infectious dose of noroviruses and the large quantities of virus in feces and vomit, contamination of foods by a single food-handler can result in large outbreaks.

Outbreaks of norovirus infections are also frequently reported in institutional settings such as nursing homes, child care centers and hospital units. In some instances, the outbreak is initially caused by a common-source exposure to a fecally contaminated vehicle such as food or water. Later, the spreading of the outbreak through person-to-person transmission among residents was facilitated by the enclosed living quarters and reduced levels of personal hygiene: because of incontinence, immobility, or reduced mental alertness. Lopman et al. demonstrated that each hospital unit (or ward) in England had 1.33 outbreaks in a one-year

period. To control the spread of the disease, hospital units were closed to new admissions. The units closed within the first 3 days of an outbreak could be re-opened earlier than those closed after day 4 or not closed at all (7.9 vs. 15.4 days). They also found that a patient who spent a year in the hospital would have an 80% chance of having a case of gastroenteritis during an outbreak. Full-time hospital staff members had a 17% chance of being affected during the follow-up year. Norovirus was the predominant etiologic agent detected in 63% of hospital unit outbreaks (followed by *C. difficile* and Rotavirus)⁸². In Hungary, there were 50 outbreaks in 2003, 61 outbreaks in 2005, 188 outbreaks in 2006, 54 outbreaks in January, 2007 and 138 outbreaks were documented in 2008⁸³.

Between 2003 and 2011, we found only 10 norovirus accumulations in hospital units. The accumulation affected those hospital wards where the personal hygiene was lower. The highest accumulation was detected at the two Internal Medicines where 3 units were affected: Gastro-Hepatology, Hematology and the General Ward. Thanks to the strict rules and separations, no epidemics were observed at the Albert Szent-Györgyi Clinical Center.

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I.

SHORT COMMUNICATION

Sporadic norovirus infections among hospitalized and non-hospitalized 0–3-year-old infants

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Abstract

Little is known of the frequency of norovirus infections in sporadic gastroenteritis cases. In a 4-y study, we examined 1152 rota-, adeno- and astrovirus negative stool samples for human noroviruses; 187 (16.2%) proved to be positive. Stool samples were from paediatric patients with acute gastroenteritis. All isolates were verified as sporadic cases: there was no accumulation.

Introduction

Human noroviruses are common aetiological pathogens that cause winter epidemics and outbreaks of acute, non-bacterial gastroenteritis in children and adults worldwide [1–4]. In Hungary, 50–100 calicivirus epidemics acquired in communities have been registered in the last 5 y. 37% of the cases of gastroenteritis were caused by HuCVs. The Hungarian data demonstrate that one-third of HuCV epidemics are observed in healthcare units [5]. There have been only a few reports on the presence of noroviruses in sporadic cases [2–3,6–8]. The aim of this study was to reveal the importance of sporadic cases in paediatric healthcare units. Due to constant control of gastroenteritis, there were neither norovirus accumulations nor epidemics in different paediatric units.

Materials and methods

Between 1 January 2004 and 31 March 2007, 1152 stool samples were collected from children aged 0–3 y. Samples were sent from the Paediatric Isolation Ward (182), the In-patient Paediatric Ward (636) and the Outpatient Paediatric Ward (146) at the University of Szeged and from paediatric patients of family doctors (188). In all samples from family doctors and outpatients, the diagnosis was acute, non-bacterial gastroenteritis. The main symptom was diarrhoea, followed in frequency by vomit-

ing. In some cases, fever, abdominal pain and a feeling of discomfort were also present.

Conventional RT-PCR was performed with the primer pairs designed by Jiang (p289 and p290/a). Through the use of these primers, it is possible to detect both noroviruses and sapoviruses [9]. During the 4 y of the present investigation we did not find any sapoviruses with these primer pairs. Real-time PCR with the primer pairs designed by Jiang and/or with our own, newly-designed primer pair (currently in the process of publication), and the Norovirus I/II antigen-ELISA kit (DakoCytomation), Norovirus I and II antigen-ELISA kit (IDEIATM) methods were used for norovirus diagnosis. Not all samples were diagnosed by using all methods. Some of the positive samples were confirmed by sequence analyses. The human rotavirus and adenovirus diagnosis was carried out with enzyme immunoassay.

Biostatistical software (SPSS15 for Windows) was applied to compare mean ages and gender distributions in the 2 groups of positive and negative test results (2-sample *t*-test and χ^2 test).

Results

Of the overall 1152 stool samples, 187 (16.2%) proved positive for noroviruses (either with PCR or with ELISA). Of the 182 stool samples from the Paediatric Isolation Ward, 53 (29.1%) were positive;

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of the 636 stool samples from the Inpatient Paediatric Ward, 87 (13.7%) were positive; of the 146 stool samples from the Outpatient Paediatric Ward, 22 (15.1%) were positive; and of the 188 stool samples from family doctors, 25 (13.3%) were positive.

The mean value (SD) in the case of negative samples was 1.179 (0.97) and in the case of positive samples was 1.266 (0.98); the difference was not significant ($t=-1.11$, $df=1150$, $p=0.27$). 18.06% of the 620 males and 14.09% of the 532 females were positive.

Of the samples from 620 males and 532 female patients, 112 and 75 female samples proved positive. The difference was not significant ($\chi^2=3.31$, $df=1$, $p=0.07$).

Figure 1 and Table I demonstrate the number of HuCV-positive samples by seasons and the age-specific 'attack rate'. We examined 303 samples in 2004, 385 in 2005, 324 in 2006 and 140 in 2007. The numbers of positive samples were: 42 (13.86%), 55 (14.28%), 57 (17.59%) and 33 (23.57%), respectively.

Discussion

With RT-PCR assays as a diagnostic tool, it has been established that human noroviruses are the most common causes of gastroenteritis outbreaks worldwide [10–12]. There have been only a few reports on the presence of noroviruses in sporadic cases [2,3,6–8]. Frogatt et al. found that during the winter season noroviruses were the second most common agents of acute non-bacterial gastroenteritis [7]. During the 4 y, we examined the presence of noroviruses in sporadic gastroenteritis cases and

Table I. The incidence of human norovirus gastroenteritis in 3 age groups.

Age (y)	Negative samples	Positive samples	Total
0–1	516 (84.8%)	92 (15.2%)	608
1–2	239 (83.5%)	47 (16.5%)	286
2–3	211 (81.8%)	47 (18.2%)	258
Total	966	186	1152

found that in children aged 0–3 y, noroviruses are common causative agents of non-bacterial gastroenteritis. We also examined the presence of noroviruses in the different paediatric wards. Most positive cases came from the Paediatric Isolation Ward (29.1%), and lower numbers of positive samples from family doctors (13.3%) and from the Inpatient (13.7%) and Outpatient (15.1%) Paediatric Wards.

Several studies have found that HuCVs are second only to rotaviruses as the main causes of viral gastroenteritis in young children [2,3,6,13]. Buesa et al. studied the importance of HuCVs in sporadic cases and in outbreaks in children under 5 y of age. In sporadic cases, HuCVs were detected in 14.19%. Rotaviruses were the most frequent agents of gastroenteritis (25.3%), and astroviruses and human adenoviruses each accounted for 3.15%. The majority of HuCV outbreaks occurred either in schools (42.8%) or in nursing homes (21.4%). The rate of noroviruses as the aetiological agents of gastroenteritis outbreaks was 56% [6]. We found that noroviruses likewise play an important role in sporadic gastroenteritis cases in Hungary. 16.2% of the stool samples contained norovirus. Most positive samples came from the Paediatric Isolation Ward. In second place as the main cause of viral

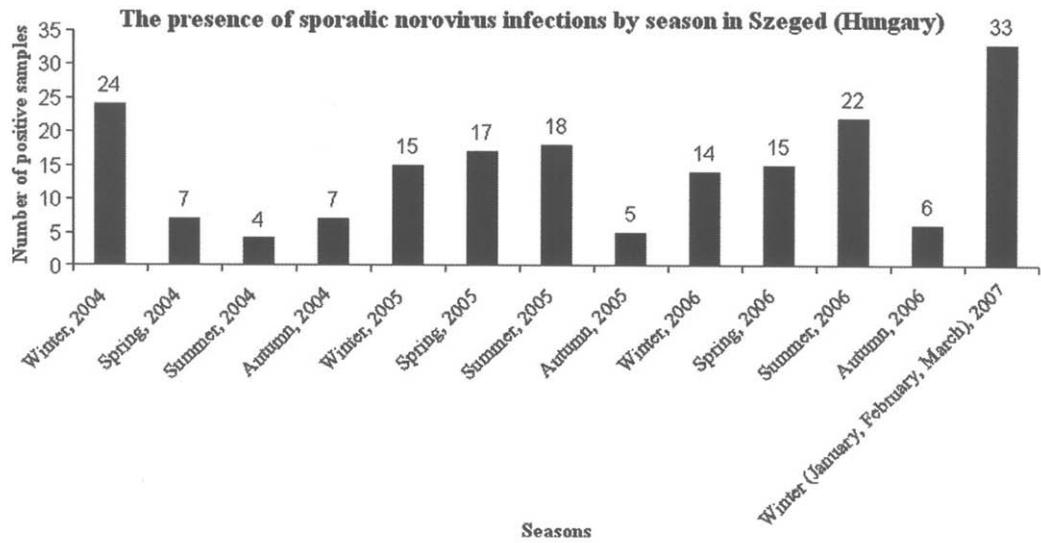


Figure 1. The presence of sporadic norovirus infections by season in Szeged (Hungary).

gastroenteritis, we found rotaviruses (10.5%), followed by human adenoviruses (4.6%). During the 4 y of the present investigation we did not find any sapoviruses.

In the 4-y study, we observed the highest number of norovirus-containing samples during the winter season and also high numbers during some of the summer seasons. We detected 42 human norovirus-containing samples in 2004, 55 in 2005, 57 in 2006 and 33 in 2007. Most samples came from the age group of 0–≤ y (52.7%); however, the incidence of norovirus gastroenteritis was the same in the 3 age groups (0–≤1, 1–≤2, 2–≤3). Owing to strict rules in Hungary, there were no norovirus gastroenteritis accumulations in the paediatric units in Szeged.

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II.



Comparison of an ELISA and two reverse transcription polymerase chain reaction methods for norovirus detection[☆]

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Abstract

Noroviruses are uncultivable; ELISA and reverse transcription polymerase chain reaction (RT-PCR) methods are therefore widely used for their detection. Sixty-one sporadic, diarrhoeal stool samples from various university hospital wards and from outpatients in Szeged, Hungary, were examined. Three methods were compared: two RT-PCR methods (the Argene Calicivirus Consensus kit and the Cepheid Norovirus Primer and Probe Set) and one ELISA method (the IDEIA™ Norovirus ELISA Test). Sensitivities of 78.9%, 92.8%, and 91.2%, and specificities of 100%, 100%, and 100% were found for the IDEIA™ Norovirus ELISA, the Argene kit, and the Cepheid kit, respectively. The PCR and ELISA systems detected 52 norovirus-positive samples, one of which belonged to genogroup I and all the others to genogroup II. Although the ELISA kit has a lower sensitivity compared to the PCR ones, it can be useful for large-scale testing. However, ELISA-negative outbreaks should be retested by RT-PCR methods. Our results suggest that noroviruses, and predominantly genogroup II of the norovirus genera, play an important role in outbreaks and sporadic cases of acute gastroenteritis, not only in infants and young children, but also in adults.

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Keywords: Comparative assay; Norovirus; RT-PCR; Sporadic cases; ELISA

1. Introduction and objectives

Outbreaks and sporadic cases of nonbacterial gastroenteritis are caused by human caliciviruses (HuCVs) in children and adults worldwide (Bon et al., 1999; Kele et al., 2009; Vinjé et al., 1997). HuCVs belong to the Caliciviridae family and are divided into four genera: *Norovirus*, *Sapovirus*, *Vesivirus*, and *Lagovirus*. The last two of these genera naturally infect only animals (Green et al., 2000). Norovirus infections generally cause nausea, vomiting, acute diarrhoea, and fever. Vomiting is more common among children, whereas diarrhoea is more likely to predominate among adults. The symptoms usually resolve within 2–3 days, although the median duration of the illness can be 4–6 days (Patel et al., 2009).

Sixty-one diarrhoeal stool samples were collected and examined for the presence of noroviruses by means of reverse transcription polymerase chain reaction (RT-PCR) methods (real-time and/or multiplex PCR) (Patel et al., 2009; Rohayem et al., 2004; Trujillo et al., 2006; Vinjé et al., 2003) and antigen ELISA (Gray et al., 2007). We decided to use the IDEIA™ Norovirus ELISA kit, because in earlier studies it was shown to have the highest sensitivity compared to other ELISA systems (Burton-Macleod et al., 2004; de Bruin et al., 2006). To the best of our knowledge, this is the first comparative study of the Argene and the Cepheid RT-PCR kits.

2. Material and methods

2.1. Stool samples

Samples were collected from infants, children, and adults (from the paediatric patients of family doctors and from eight different wards at the University of Szeged) with clinical symptoms of acute gastroenteritis: nausea, vomiting, and/or

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three or more loose stools in 24 h. Exclusion criteria were rotavirus, adenovirus, or other enteral positivity. One stool sample was collected per patient during the acute phase of the infections; samples were stored at +4 °C until processing.

2.2. Laboratory procedure and methods

For norovirus detection, two RT-PCR methods were used (The Argene Calici/Astrovirus Consensus kit [Argene, North Massapequa, NY, USA] and the Cepheid Norovirus Primer and Probe Set real-time RT-PCR [Cepheid, Sunnyvale, CA, USA]) and one ELISA method (the IDEIA™ Norovirus ELISA, DakoCytomation, Ely, UK).

2.3. RNA extraction

A stool suspension from 10% to 50% in 1 mL sterile phosphate buffered saline was performed. The sample was centrifuged at 12,000 × g for 15 min. The supernatant was collected. Viral RNA was extracted by using the QIAamp Viral RNA Mini Kit (Qiagen, Victoria, Australia, Ref. No. 67-120A) according to the manufacturer's instructions. This protocol was performed for the purification of viral RNA from a 140-µL stool sample; the elution volume was 60 µL. Extracted viral RNA was stored at -70 °C.

2.4. Reverse transcriptase polymerase chain reaction

The RT-PCR methods were carried out as specified by the manufacturers' instructions. The Argene Calici/Astrovirus Consensus kit is a PCR system used in combination with an ELISA method. The kit allows specific genome detection of all HuCVs (norovirus and sapovirus genera). After RNA isolation, reverse transcription, and amplification steps, HuCVs are detected with a microtitre plate, using hybridization of their amplified product with a biotinylated probe. The kit also contains an internal control. For each amplification run, 10 µL of extracted RNA was used. The time required is 1.5 days. The Cepheid Norovirus Primer and Probe Set consists of two types of beads: norovirus beads and sample preparation control (SPC) beads. Norovirus beads contain primers and two FAM-labeled probes for the detection of genogroup I (GGI) and one Alexa Fluor (Molecular Probes, Inc., Eugene, OR, USA) 647-labeled probe for the detection of genogroup II (GGII). The norovirus beads also contain primers and a Texas Red-labeled probe for a separate sample preparation control sequence. The SPC beads contain an RNA target for sample preparation control. By using different hybridization probes, we can differentiate between the norovirus genotypes GI and GII. With the Cepheid Norovirus Primer and Probe Set, 5 µL of extracted sample was used for each real-time PCR run and the time required was approximately 6 h (depending on the number of samples being examined).

2.5. IDEIA™ Norovirus ELISA system

This kit is an enzyme immunoassay for the qualitative determination of noroviruses of GGI and GGII in stool

samples. The IDEIA™ Norovirus ELISA system utilizes wells coated with GGI- and GGII-specific monoclonal antibodies. It is a very simple, rapid, but labour-intensive method for simultaneous screening of a large number of samples. The ELISA takes approximately 2 h to carry out.

2.6. Statistical analysis

The kappa coefficient was calculated to determine the level of agreement between assays (IDEIA™ Norovirus ELISA versus Argene Calici/Astrovirus Consensus kit, IDEIA™ Norovirus ELISA versus Cepheid Norovirus Primer and Probe Set, Cepheid Norovirus Primer and Probe Set versus Argene Calici/Astrovirus Consensus kit), and sensitivity, specificity, positive (PPV), and negative (NPV) predictive values were determined. A patient was adjudged to be infected with norovirus if the stool sample was positive using one of the two RT-PCR methods, because the RT-PCR offers the ability to detect lower levels of the virus compared to ELISA systems.

3. Results

During the study, we examined 61 stool samples for noroviruses. The age of the patients involved ranged between 4 days and 83 years (median age: 7.5 years; mean age: 28.0 years). The male/female ratio was 25:36. There were no cases of repeated infections during the study period.

Of the 61 samples, 52 were positive for norovirus, with 47 of the latter being true positive. The results are presented in Tables 1 and 2 and Fig. 1.

(A) The IDEIA™ Norovirus ELISA revealed 38 norovirus-positive and 23 negative samples. The kappa coefficient between the ELISA and the Argene Calici/Astrovirus Consensus kit was 0.50 (standard error [SE]: 0.12; 95% confidence interval (CI): 25–75%); the kappa coefficient between the ELISA and the Cepheid Norovirus Primer and Probe set was 0.42 (SE: 0.14; 95% CI: 14–69%). The sensitivity of the test was 78.9%, the specificity was 100%, the PPV was 100%, and the NPV was 39.1%.

Table 1
Comparison of three methods for the diagnosis of norovirus in diarrhoeal fecal samples (N = 61)

Methods	Positive	Negative	Borderline	Inhibitors
Argene Calici/Astrovirus Consensus kit*	48 (79.1)	10 (16.1)	2 (3.2)	1 (1.6)
Cepheid Norovirus Primer and Probe Set	47 (77.4)	8 (12.9)	0 (0)	6 (9.7)
IDEIA™ Norovirus EIA	38 (61.3)	23 (38.7)	0 (0)	0 (0)

Values are shown as n (%).

* The Argene Calici/Astrovirus consensus kit is suitable for human astrovirus detection. Human astroviruses were not detected in this study.

Table 2
Discrepant ELISA and PCR results

Simultaneously positive	Sample number	Sex	Ward	Age (years)	Calici/NLV ELISA	Argene Calici PCR	Cepheid Norovirus PCR
Only with 1 method							
	20	M	Outpatient	4	–	+	Inhibitory samples
	30	F	Inpatient	<1	–	+	–
	55	F	Inpatient	78	–	+	Inhibitory samples
	56	M	Inpatient	40	–	–	+
	57	M	Inpatient	<1	–	–	+
	60	F	Inpatient	23	–	–	+
With 2 methods							
	06	F	Outpatient	37	+	+	–
	46	M	Inpatient	71	+	+	–
	09	F	Inpatient	6	+	–	+
	14	F	Inpatient	16	–	+	+
	22	F	Outpatient	3	–	+	+
	37	F	Outpatient	3	–	+	+
	53	F	Outpatient	53	–	+	+
	58	M	Outpatient	<1	–	+	+
	59	M	Inpatient	<1	–	+	+
	61	M	Inpatient	38	–	+	+
	62	F	Outpatient	53	–	+	+

F = Female; M = male; inpatient = samples from inpatient ward; outpatient = samples from outpatient unit; + = positive result; – = negative result.

(B) With the use of the Argene Calici/Astrovirus Consensus kit for the 61 samples, 48 were found positive for HuCVs, 10 were negative, 2 were borderline, and 1 contained inhibitors. The sensitivity of the test was 92.8%, the specificity was 100%, the PPV was 100%, and the NPV was 69.2%. The kappa coefficient between the Argene Calici/Astrovirus Consensus kit and the Cepheid Norovirus Primer and Probe set was 0.55 (SE: 0.14; 95% CI: 27–82%).

(C) With the Cepheid Norovirus Primer and Probe Set, 47 of the 61 stool samples proved to be positive for human noroviruses; 46 were GII and only 1 was GGI positive. Eight samples were negative and only

6 contained inhibitors. The sensitivity of the test was 91.2%, the specificity was 100%, the PPV was 100%, and the NPV was 64.3%.

4. Discussion

By using different RT-PCR methods, we were able to characterize and identify norovirus epidemics. In clinical practice, it is important to obtain results as soon as possible in order to minimize the spread of the virus within and between hospital units. The high genetic and antigenic diversity poses problems in the laboratory diagnosis of noroviruses; in this study, therefore, we compared 3 different norovirus diagnostic methods. The PCR detects the viral genome, while the ELISA is directed against the viral capsid proteins (antigen). The detection limit for PCR is very low (depending on the PCR efficiency and assay optimization). In an earlier study, we showed that the viral load in norovirus-infected samples is very high (in press). In many cases, stool samples contain a high number of PCR inhibitors. The detection limit for ELISA assays is currently estimated as 10^4 to 10^5 particles per millilitre.

By using the QIAamp Viral RNA Mini kit, we effectively eliminated PCR inhibitors. The number of inhibited PCRs was only 1 (1.61%) with the Argene Calici/Astrovirus Consensus kit and 6 (9.68%) with the Cepheid Norovirus Primer and Probe Set. The Argene RT-PCR was carried out in 50 μ L, while the Cepheid RT-PCR only in 25 μ L, so the inhibitor could have been overdiluted in the Argene mastermix, whereas in the Cepheid it could not; this resulted in more inhibitory samples. We used different recombinant polymerases in the different PCRs; the polymerases differ from one another in their sensitivity. This may explain the different number of inhibited samples.

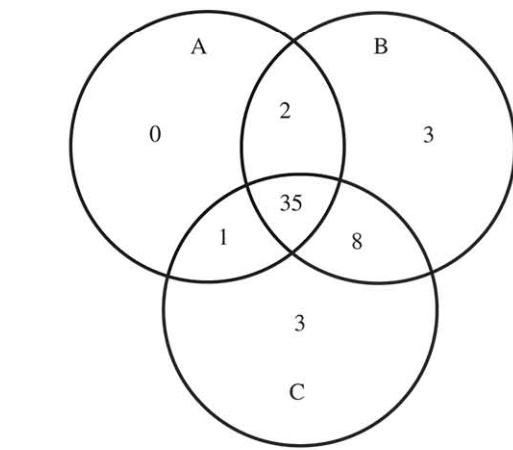


Fig. 1. (A) Number of positive samples with the IDEIA Norovirus ELISA. (B) Number of positive samples with the Argene Calici/Astrovirus Consensus kit. (C) Number of positive samples with the Cepheid Norovirus Primer and Probe Set.

We found 14 RT-PCR-positive samples, which were negative by ELISA. This can be due to the fact that the detection of norovirus in stool samples by ELISA is based on the detection of viral antigens and may thus be hampered by the antigenic diversity of noroviruses. Three samples were positive by using the Cepheid kit but negative with the Argene kit. This can be explained by the fact that the Cepheid RT-PCR is a real-time PCR, with fluorescent probes and its sensitivity is much higher than that of commercial PCRs. Three samples were positive with the Argene kit and negative with the Cepheid kit and ELISA. These 3 samples might have contained Sapporo virus RNA.

ELISA systems are fast and use a simple method for detecting norovirus antigen in stool samples. With these systems, we were able to screen a large number of samples at the same time. The assays are not suitable for the diagnosis of infection in individual patients or for genotype assignment. RT-PCR assays are a powerful alternative method for the laboratory diagnosis of nonbacterial, acute gastroenteritis (Parashar and Monroe, 2001). Furthermore, genome amplification allows the molecular characterization of viral strains. We found that the most sensitive and specific method is the Argene Calici/Astrovirus Consensus kit and it can be useful also in clinical and research settings.

Ninety-nine percent of the positive samples belonged to GGII and only 1 to GGI. This is consistent with the findings of other studies of sporadic cases of gastroenteritis in children (Buesa et al., 2002; Kirkwood et al., 2005; Lau et al., 2004). Norovirus GGI strains constitute a minor proportion of strains in most outbreak studies (Kirkwood et al., 2005; Frankhauser et al., 2002; Guo et al., 2007; Koopmans et al., 2001). Our results suggest that noroviruses, and predominantly GGII, play an important role in both sporadic cases and outbreaks of acute gastroenteritis, not only in infants and young children, but also in adults.

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III.

Sporadicusan előforduló humán calicivirusok kimutatása molekuláris genetikai módszerekkel

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Összefoglalás

A sporadicusan előforduló humán calicivirusok diagnosztikai lehetőségeit vizsgáltuk. A korábban Jiang által leírt primer párral történő PCR mellett a real-time PCR módszer kifejlesztése és egy új primer pár tervezése és bevezetése volt a cél. 2003. január 1.-je és 2005. január 1.-je között 884 székletminta vizsgálata történt meg a Szegedi Tudományegyetem, Általános Orvostudományi Kar, Klinikai Mikrobiológiai Diagnosztikai Intézetében. Elemeztük a humán calicivirus nucleinsavat tartalmazó minták nemek és korcsoport szerinti megoszlását. Új primer párt terveztünk, mellyel 110 véletlenszerűen kiválasztott minta vizsgálata valósult meg, melyek közül 37 (33,6%) bizonyult humán calicivirus sequentiának. Ugyanezen 110 mintából a Jiang-féle primer párral 6 (5,4%) mintában találtunk humán calicivirus specifikus nucleinsavat. Az eredményeink alapján a valós-idejű PCR módszer kiváló diagnosztikai eszköznek bizonyult, mert segítségével gyorsabb, és sensitívabb vizsgálatok valósíthatók meg, mint a hagyományos PCR módszerrel. A humán calicivirusok nagymértékű genetikai variabilitása miatt a molekuláris diagnosztika legnagyobb nehézsége tervezni egy olyan primer pár, melyel a vizsgálatot egyidejűleg specifikussá és sensitívvé tehetjük. A diarrhoeás esetek 79%-ában a virális eredetet nem sikerült bebizonyítanunk.

Bevezetés

A humán calicivirusok (HuCV) az acut, nem bacterialis gastroenteritisek gyakori kórokozói világszerte, függetlenül az életkortól. A fertőzés forrása lehet a széklettel contaminált víz, élelmiszer, a közvetlen kontaktus és a vírus aerosol útján is terjedhet.⁽¹⁾ Szepetembertől májusig gyakoribbak a calicivirusok által

okozott megbetegedések, jelenlétékkel azonban egész évben számolni kell. Járványok általában időlegesen zárt közösségekben alakulnak ki (kórházak, óvodák, iskolák, idősek otthona), aholával bekerülve primer és secundaer módon nagyon gyorsan terjed a fertőzés és rövid idő alatt nagyszámú megbetegedést okozhat.

HuCV-k 27-40 nm átmérőjű, ikozahedrális szimmetriájú, burok nélküli particulumok, melyeknek genetikai információtartalmát pozitív egyszálú RNS hordozza.⁽⁷⁾ Kezdetben a taxonómia morphologiai hasonlóságokon és különbségeken alapult, ma már a genetikai sequentia- és filogenetikai analízis a méravadó. A *Calicivirusidae* családba négy genus tartozik: *Lagovirusok*, *Vesivirusok*, *NLVs* (Norwalk-szerű vírusok) és *SLVs* (Sapporo-szerű vírusok). Az első két genusba tartozó vírusok kizárolag állatokat betegítének meg. A NLV-k hivatalos neve régebben 'kis kerek struktúrájú vírusok volt' (SRSVs, Small round structured viruses), az NLV és SLV ideiglenes elnevezés, névváltoztatás várható.⁽⁶⁾

Az NLV nemzettség filogenetikai szempontból legkevésebb két ágra oszlik (GG1 és GG2 genocsoport) a polymerase-ban és a capsid régióban fellelhető genetikai különbségeknek köszönhetően. A genocsoporton belüli további alosztályokra való bontásra még nincs egyértelmű szabály, elnevezésükelfedezési helyük alapján történik. A mai álláspont szerint hozzávetőleg hétek GG1 (Norwalk, Southampton, Desert Shield, Chiba, Winchester, Saitama, Sindlesham) és tíz GG2 (Snow Mountain, Toronto, Bristol, Hawaii, Mexico, Grimsby, Melksham, Lordsdale, Camberwell, White River) csoport létezik. A CDC kutatói szerint létezik egy harmadik, a GG3 genocsoport, melyhez állatokat fertőző vírusok (Jena és Newbury ágens-2) tartoznak, de ezek taxonómiai helyzete még nem tisztázott.⁽¹⁾ A SLV-k is két genocsoportra oszthatók. Az első genocsoportba tartozik a névadó, Sapporo vírus, míg a második genocsoportba a London vírus sorolható.⁽⁸⁾

Az inkubációs idő viszonylag rövid, 12-60 óra. A betegeknél tipikus tünetek figyelhetők meg: diarrhoea, abdominalis fájdalom és hányinger. Felnőtteknél a hányás a vezető tünet, ami ez által megkülönbözteti a NLV-t más virális és bacterialis kórokozóktól, például *Salmonella*-, *Shigella*-, *S. aureus* okozta fertőzésekkel. A NLV járvány kitörésének leggyorsabb felismerése a

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Rövidítések: HuCV: humán calicivirus; NLVs: Norwalk-szerű vírusok; SLV: Sapporo-szerű vírusok, RT-PCR: revers transkriptióhoz kapcsolt polymerase láncreakció

klinikai tünetek és epidemiológiai jellemzők közti összefüggések észrevételen alapul. A betegség lefolyása gyors, átlagosan 24-48 órán át tart.⁽⁹⁾

A NLV-k és a SLV-k leggyakrabban oralis úton jutnak be a szervezetbe. Savstabil vírusként ájtutnak a gyomron, bekerülnek a vékonybélbe, ahol megkezdtődik a replicatio.⁽⁶⁾ A calicivirus infekció gyanúja akkor merül fel, ha a székletmintába bacteriológiai tenyésztésének eredménye negatív, az átlagos inkubációs idő 24-48 óra, a betegség lefolyása gyors, 12-60 óra és az esetek >50%-ában a hányás a vezető tünet. Európában a calicivirus fertőzések száma október, november elején kezd emelkedni, maximumát januárban, minimumát május, június táján éri el. A calicivirus fertőzés lehetősége életkortól független.⁽⁷⁾

Jelen munkánk során a cél az volt, hogy bevezessünk egy olyan új, humán calicivirus diagnosztikára alkalmas módszert, melyel a vírus kimutatása az eddiginél gyorsabban és pontosabban megvalósítható.

Anyagok és módszerek

2003. január 01.-je és 2005. január 01.-je között a Klinikai Mikrobiológiai Diagnosztikai Intézetbe küldött székletmintákat dolgoztuk fel. A mintákból az intézetünkben alkalmazott diagnosztikai algoritmusoknak megfelelően először rota- és adenovírus antigén vizsgálatot végeztünk. Korábbi tapasztalataink szerint a rotavírus fertőzés fordul elő leggyakrabban, valamint az általunk használt gyorsdiagnosztikai kit a két vírusantigént egyidejűleg mutatja ki. Amennyiben ezek a vizsgálatok negatív eredménnyel zártultak, a székletmintákból astrovírus antigén jelenlétéit kíséréltek meg kimutatni. Ha ez a vizsgálat is negatív eredménnyel zártult, akkor végeztük el a calicivirus RT-PCR vizsgálatot.

A virális nucleinsav isolálására a V-gene kiteket használtuk (Building B2-1; Xiacheng Industrial Zone (Huafeng, Shiqiao); Hangzhou 310022, P. R. China). A Norwalk vírus nucleinsavának amplifikációja revers transcriptióhoz kapcsolt polymerase láncreakcióból történt.^(2,3) A revers transcriptio során az RNS-t cDNA-sé írtuk át. minden vizsgálatnál 47,5 µl master mixet mértünk az eppendorf csöbe, melynek összetétele: 0,14 U/µl M-MuLV-RT (Fermentas); 0,2 U/µl rRNasin (Fermentas); 3 ng/µl p289 [5'-TGACAATGTAATCATCACCATA (nt pozíció: 4865-4886)]; 0,4 mM dNTP; 0,5 mM MgCl₂ (SIGMA, Saint Louis, USA); 10xPCR Buffer [100 mM Tris-HCl (pH: 8,3), 15 mM MgCl₂, 500 mM KCl, 0,01% gelatin; SIGMA, Saint Louis, USA]; RNase mentes víz (Promega, Madison, USA). Ehhez a keverékhez adtunk 3 µl tisztított RNS-t, majd a csöveket egy órán át 42 °C-on inkubáltuk. Ezután a mintához hagyományos PCR esetén hozzáadtunk eppendorf csövenként 50 µl PCR master mixet. Az amplifikációs elegy összetétele a következő volt: RNase mentes víz (Promega, Madison, USA); 10xPCR Buffer [100 mM Tris-HCl (pH: 8,3), 15 mM MgCl₂, 500 mM KCl, 0,01% gelatin; SIGMA, Saint Louis, USA]; 0,5 mM MgCl₂ (SIGMA, Saint Louis, USA); 0,1 U/µl Taq polymerase és 3 ng/µl p290/A

primer (a primer nucleotid sorrendje publikálás alatt áll). Az amplifikációt Perkin Elmer GeneAmp PCR System 9600-as készülékkel végeztük. A PCR reakció során az elődenaturáció 3 percen át tartott 94 °C-on, majd a 40 ciklusú polymerase láncreakció következett, melynek paraméterei: 94 °C 1 perc, 37 °C 2 perc és 72 °C 1 perc. A ciklusok végeztével a végső extensio 10 percen át, 72 °C-on zajlott. A termékeket tartalmazó eppendorf csöveget 4 °C-on helyeztük el, az amplifikált termékek gélelectrophoresissel történő vizsgálatáig.⁽¹²⁾ Az amplifikált termékek PCR utáni vizsgálatát agarose gélectrophoresissel végeztük. A vizsgálathoz 1,5%-os agarose (Top Vision, Fermentas) gélt használtunk. Az electrophoresist egy órán át, 130 volton és 60 mA-en végeztük. A detektálás ethidium-bromid segítségével UV-fény alatt történt, a gélekről Kodak EDAS 290-es gélfotó berendezéssel fotót készítettünk, melyet a Kodak 1D 3,5-ös program segítségével vizsgáltunk.

A kétlépéses valós-idejű PCR alkalmazása estén a revers transcriptiót a már leírt módon végeztük, majd az amplifikációt a LightCycler (Roche Diagnostics GmbH D-68298 Mannheim, Germany) amplifikáló és detektáló készülékben valósítottuk meg. A 10 µl végtér fogatú amplifikációs elegy összetétele, mely 1 µl templátot tartalmazott: RNase mentes víz, 2,5 mM MgCl₂ (SIGMA, Saint Louis, USA), 10x Master Mix SYBR Green-nel (Roche Diagnostics GmbH D-68298 Mannheim, Germany), 20 ng/µl p289, 20 ng/µl p290/A. Új primer párral történő diagnosztikánál a revers transcriptio során alkalmazott körülmenyek nem változtak, az alkalmazott primer kivételével. Az amplifikációs elegy összetétele a korábban használattól az csupán a primerek mennyiségében különbözött. Optimálisnak mind a forward, mind a revers primer esetén a 1,2 pM-os mennyiség bizonyult, 10 µl végtér fogatra számolva.

Eredmények

2003. január 1.-je és 2005. január 1.-je között 884 székletmintá vizsgálata történt meg a Szegedi Tudományegyetem, Általános Orvostudományi Kar, Klinikai Mikrobiológiai Diagnosztikai Intézetében. Rotavírus antigént 56 esetben (6,3%), adenovírus antigént 36 esetben (4,1%), astrovírus antigén jelenlétéit 16 esetben (1,8%) mutattuk ki. A fennmaradó 645 mintából 77 esetben (11,9%) sikerült humán calicivirus kimutatni (1. ábra). A hasmenéses esetek 79%-ában a virális eredetet nem sikerült bebizonyítanunk.

2003. január és 2003. december között 142 esetben hagyományos PCR vizsgálat történt a Jiang által leírt primer párral. A 142 mintából 12 (8,5%) esetben sikerült humán calicivirus kimutatnunk. 2004. január hónapban sikeresen adaptáltuk a Jiang-féle primer párral történő HuCV diagnosztikai módszert a valós-idejű PCR-hez. 2004. október hónapig 400 mintát dolgoztunk fel, ezek közül 50 (12,5%) bizonyult calicivirus sequenciának.

1. táblázat. HuCV vizsgálatok beküldők szerinti megoszlása

	2003.01.01.-2003.12.31.		2004.01.01.-2004.12.31.	
	Vizsgált minták (N)	HuCV nucleinsavat tartalmazó minta (N)	Vizsgált minták (N)	HuCV nucleinsavat tartalmazó minta (N)
SZTE, Gyermekklinika ambulanciái	156	5	106	15
SZTE, Gyermekklinika osztályai	114	9	267	19
SZTE, Gyermekklinika PIC	20	0	14	3
SZTE, Gyermekkórház	5	0	3	0
Körzeti gyermekgyógyászati rendelők	58	1	82	10
SZTE, Családorvosi Intézet	13	8	11	1
SZTE, Nőgyógyászati Klinika	12	2	2	0
SZTE, Bőrgyógyászati Klinika	3	0	2	0
SZTE, II. Belgyógyászati Klinika	0	0	7	2
SZTE, I Belgyógyászati Klinika	0	0	2	0
SZTE, Igazságügyi Orvostani Intézet	2	0	2	1
SZTE, Fül-Orr-Gégészeti Klinika	0	0	1	1
SZTE, Traumatológiai Klinika	1	0	0	0
SZTE, Szemészeti Klinika	1	0	0	0
Összesen	385	25	499	52

2. táblázat. 2003. január 1.-je és 2005. január 1.-je között Intézetünkbe érkezett rota-, adeno- és astrovirust nem tartalmazó minták HuCV vizsgálatának eredménye korcsoport szerinti bontásban

Évek	Életkor (év)																	
	0-3		4-6		7-9		10-12		13-15		16-18		19-24		25-29		>29	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
2003	8	71	5	11	1	13	1	8	1	6	0	3	1	5	2	11	6	25
2004	30	243	5	38	2	25	5	20	0	17	3	13	0	9	6	8	1	20

3. táblázat. HuCV pozitív minták korcsoport és nemek szerinti megoszlása

Évek	Életkor (év)																	
	0-3		4-6		7-9		10-12		13-15		16-18		19-24		25-29		>29	
	Férfi	Nő	Férfi	Nő	Férfi	Nő	Férfi	Nő	Férfi	Nő	Férfi	Nő	Férfi	Nő	Férfi	Nő	Férfi	Nő
2003	7	1	2	3	1	0	1	0	1	0	0	0	1	0	0	2	3	3
2004	19	11	2	3	1	1	3	2	0	0	0	3	0	0	2	4	0	1

Ezen időszakban vezettük be az új, általunk tervezett primer párral történő valós-idejű PCR diagnosztikát, mellyel 125 minta meghatározása történt 2004. decemberréig. A 125 mintából 21 (16,8%) esetben sikerült calicivirus nucleinsavat kimutatnunk. Összehasonlító vizsgálatot végeztünk 110 véletlenszerűen kiválasztott mintából a Jiang-féle és az újonnan tervezett primerekkel. A Jiang-féle primer párral 6 (5,4%) minta bizonyult humán calicivirus sequentiának, az új primerekkel 37 (33,6%) minta esetén mutattuk ki a HuCV nucleinsavat. Mindez annak köszönhető, hogy az általunk tervezett primerek segítségével többnyire az európai calicivirus törzsek diagnosztizálhatók, míg a Jiang által tervezettek 'univerzálisabbak', velük többféle törzs felismerhető, mely azonban magában hordozza a nagyobb hibalehetőséget. A HuCV pozitív mintákat 13 esetben sequenáltuk, hogy a vizsgálatunk specifikusságát kontrollálhassuk. A sequenáltatott minták minden esetben Norwalk-szerű vírusok közé tartoztak.

A vizsgálatkérő intézetek szerinti megoszlást az 1. táblázatban foglaltuk össze. A legtöbb kérés a Szegedi Tudományegyetem Gyermekklinikájának osztályairól és ambulanciáiról érkezett. Ennek megfelelően a legtöbb, sporadicusan előforduló humán calicivirus a 0-3 éves korosztály mintáiból, de a többi korcsoport mintáiból is sikerült a vírust, mint kórokozót azonosítanunk. A HuCV nucleinsavat tartalmazó minták korcsoport szerinti megoszlását a 2. táblázatban tüntettük fel.

Megvizsgáltuk a humán calicivirus specifikus nucleinsavat tartalmazó minták nemek szerinti megoszlását (3. táblázat). Csecsemőkorban több kisfiú betegedett meg humán calicivirus által okozott fertőzésben, mint leány. A további korcsoportokban a nemek fertőzöttségi aránya kiegyenlítődést mutat.

Megbeszélés

2003. január elseje óta végezzük Intézetünkben a humán calicivirus diagnosztikát. A regionális ÁNTSZ-ekkel szemben mi sporadicus esetekből is megpróbáltuk diagnosztizálni a vírust, mivel egy közösségen, családban, melyben egyszer kimutatható a vírus, nagy valószínűséggel halmozódott előfordulást és járványt is okozhat. A hagyományos PCR-el, a Jiang által leírt primer párral, az esetek 8,5%-ában sikerült bizonyítanunk a vírus károkozó szerepét. Sikeresen adaptáltuk a valós-idejű PCR módszert, melynek segítségével már a minták 12,5%-ában találtunk humán calicivirus specifikus nucleinsavat. A két módszer nemcsak a sensitivitásban, hanem a diagnosztikához szükséges időben is különbözik. A valós idejű PCR során az amplifikációhoz szükséges idő lényegesen lerövidül. Ennek főbb oka volt az amplifikatiós ciklusok kevesebb száma; az amplifikatio szili-kaüveg capillarisokban történt, melyek sokkal rövidebb idő alatt melegíthetők/hűthetők, mint az eppendorf csövek, a capillarisok olyan hőblokkban foglalnak helyet, mely sokkal gyorsabban képes változtatni hőmérsékletét, mint a hagyományos PCR készülék és nincs szükség gél-electrophoresisre, mert a termékek analízise fluorescens detectálással történik.⁽¹⁰⁾ A post-PCR termék vizsgálata melting point analízis (olvadáspont analízis) segítségével történt. Vizsgálataink során derült fény arra, hogy a Jiang által leírt primer párok használatával a hagyományos és a valós-idejű PCR-ek esetén gyakran aspecifikus termékek mutathatók ki (pl.: humán 11. chromosoma részlet, astrovirus- és enterovirus nucleinsav). Ezért tüztük ki célul, hogy új primer párt tervezünk egy számítógépes program segítségével, mellyel a calicivirus diagnosztika az eddigieknel sensitívebbé és specifikusabbá válhat. Az általunk írt számítógépes programmal szemben támasztott kritériumaink a következők voltak: 1. legalább 10-12 nucleotidból álló régiót keressen a program, mely minél több eddig publikált sequenciában fellelhető, 2. a program gyűjtse ki az összes, a kritériumoknak megfelelő szakaszokat és rendezze egy adatbázisba. Kiválasztottuk ezek közül a legspecifikusabb forward primert, ennek az olvadáspontjához közel azonos revers primert kerestünk, melynek specificitása hozzá hasonló. A pozitív kontroll olvadáspontja a Jiang-féle primer párral 85,7 °C, az általunk tervezett primer párral dolgozva: 81,5 °C. Az új primerekkel 125 mintából 21 (16,8%) esetben sikerült humán calicivirus specifikus nucleinsavat kimutatnunk.

2004-ben Pang és munkatársai is kifejlesztettek egy valós-idejű PCR módszert, mellyel a Norwalk-szerű vírusok detektálhatók. Járványokból és sporadicus esetekből származó székletmintákkal dolgoztak. minden minta, mely a hagyományos PCR-rel pozitívnak bizonyult, a valós-idejű PCR-rel is ugyanazt az eredményt adta. Vizsgálataik során a valós-idejű PCR módszer érzékenyebbnak bizonyult, mint a hagyományos PCR. Mennyiségileg is meghatározta a kimutatható RNS számot, ennek értéke 5-5*10⁶ kópia RNS/reakció, ami 25.000 kópia RNS/ széklet grammot jelent.⁽¹¹⁾

A Norwalk-szerű vírusok diagnosztikájára alkalmas módszert fejlesztettek ki Kageyama és munkatársai. 37 járványból 71 székletmintá bizonyult Norwalk-vírus sequentiának. Az amplificált nucleinsavat sequenáltatták, majd a legkonzervatívábbnak bizonyuló régióra új primer párt és TaqMan próbát terveztek, mellyel minden a két genocsoport (GG1 és GG2) detektálható. Az elektronmikroszkóppal pozitívnak bizonyuló minták 99%-ából sikerült Norwalkszerű vírust kimutatniuk, továbbá 20, előzőleg elektronmikroszkóppal negatívnak bizonyult mintában találtak Norwalk-vírus sequentiákat. A vírus kimutathatóságát 10 kópia/reakcióban állapították meg.⁽⁵⁾

Pang és munkatársai 132, Kageyama és munkatársai 110 székletmintát dolgoztak fel és vizsgáltak meg real-time PCR módszerrel, amely csak a Norwalk-szerű vírusok diagnosztikájára alkalmas. Az elmúlt két évben összesen 542, sporadicusan előforduló mintát vizsgáltunk meg a Jiang-által tervezett primerekkel, hagyományos PCR módszerrel, valamint az általunk bevezetett és kifejlesztett real-time PCR-el mely egyidejűleg alkalmas minden a NLV-ök és minden a SLV-ök kvalitatív kimutatására. Az általunk tervezett primer párral történő valós-idejű PCR-rel az esetek 16,8%-ában sikerült a Norwalk-szerű vírust, mint károkozót azonosítanunk. Az új primerekkel megvalósuló, valós-idejű PCR módszerrel a Norwalk-szerű vírusok diagnosztikáját az eddigieknel sensitívebbé és specifikusabbá sikerült tennünk.

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Irodalom

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Kele, B., Somogyvári, F., Deák, J.: Diagnostic of sporadic occurring human caliciviruses by using molecular genetic methods

Summary

Our objectives were to develop an assay for the detection of human caliciviruses by using the LightCycler SYBR green real-time PCR method with previously published primers called p289 and p/290a and the newly designed ones. Between 1st January, 2003 and 1st January, 2005, 884 stool samples were collected from patients who were attending the University Hospital of Szeged or who presented to their family doctors. All of these were sporadic cases. We analysed the human calicivirus positive samples according to sex and age groups. We compared 110 samples with the Jiang-designed and the newly designed primers. 37 proved to be positive with the newly designed ones whereas only 6 samples were positive by using the Jiang designed primers. Real-time PCR systems are excellent diagnostic tools, because they are much more specific, sensitive and faster than the traditional PCR methods. Due to the variability of the calicivirus genome the major difficulty in molecular the assay is that it is difficult to find a primer pair, wherewith the human calicivirus diagnostic could become more specific and sensitive.