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EXAMINATION OF HEPATITIS C AND G AND ENTEROVIRUS INFECTIONS IN PREGNANT, NEWBORN, PEDIATRIC AND ADULT POPULATIONS

PhD thesis

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Summary

The profound influence of molecular techniques on all branches of biomedical science is most apparent in virology. As concerns human infectious viral diseases, the direct detection of hepatitis C virus (HCV), hepatitis G virus (HGV) and enteroviruses is based mainly on molecular diagnostic methods. These viruses are challenging problems worldwide: the number of persons infected annually is several millions. The illness caused by HCV starts with mild or nonspecific symptoms, sometimes followed by the development of clinical hepatitis. No illness has yet been associated with HGV, and fewer than 1% of enterovirus infections are associated with specific illnesses (meningitis, pleurodynia, peri- or myocarditis, conjunctivitis, hand-feet-mouth disease, flaccid paralysis, etc.). In the case of a modulated immune status, as pregnant women and newborns, the situation is more complicated. The correct diagnosis cannot be established without molecular diagnostic methods.

The aims of this study were to investigate the epidemiology of HCV and HGV infections among healthy and risk populations in South Hungary, to determine the usefulness of different microbiological methods for the detection of HCV infection, to determine the HCV genotypes of patients with chronic hepatitis C in South Hungary and to compare the resulting data with those obtained from a randomized number of patients with chronic hepatitis C in South-east Austria, to evaluate a qualitative molecular assay for the detection of enteroviruses from cerebrospinal fluid (CSF) by using automated RNA extraction instrument and real-time PCR, to investigate clinical specimens after determination of the detection limit and to compare the results with those of a qualitative in-house PCR assay.

Between 1992 and 2001, blood donors, health-care workers, pregnant women, children and patients with chronic hepatitis C in South Hungary were examined for the presence of HCV by second and third-generation enzyme-linked immune assay (ELISA), reverse-transcriptase PCR (RT-PCR) and automated sequence, phylogenetic analysis, and for the presence of HGV by ELISA. Enteroviruses were examined by real-time PCR with automated RNA extraction and compared to a qualitative in-house PCR assay in CSF samples.

Of the 45 719 blood donors tested, 195 (0.42%) were confirmed as anti-HCV-positive. Comparison of the groups revealed that those who had had more than three pregnancies, undergone surgery, transfusion, tattoos or piercing for earrings were significantly more at risk of acquiring HCV infection. A relatively low prevalence of confirmed anti-HCV-positive blood donors was found; the transmission of HCV occured mainly by nosocomial route.

Of 500 screened pregnant women, 5 (1%) exhibited anti-HCV, and in 3 of the 5 persons (0.6%) HCV RNA was found. Until confirmation of the effectivity of elective cesarean section for preventing HCV transmission, the screening of pregnant women for HCV infection is not recommended. Of 120 children who had received blood transfusions prior to the implementation of anti-HCV blood donor screening, 2 (1.7%) were found to be confirmed anti-HCV-positive. In the control pediatric population (50), no sign of HCV infection was found. Of 2 051 health-care workers 11 (0.53%) were confirmed anti-HCV-positive, and 10 were positive by RT-PCR, but in 3 further cases anti-HCV positivity was confirmed by RT-PCR only. Because of the low prevalence of anti-HCV-positive blood donors, pregnant women and health-care workers in South Hungary, only a very small number of children, who had received blood transfusions prior to implementation of the anti-HCV blood donor screening, were infected with HCV.

In all cases (20) examined, genotype 1 was found, with dominance of the 1b subtype in South Hungary. Comparison with genotyping results from South-east Austria, where 15 patients infected with genotype 1 were found and the remaining 5 patients were infected with either genotype 3 or genotype 2, reflects the present situation regarding the prevalence of the HCV genotypes in South Hungary and South-east Austria, with genotype 1 being the predominant subtype in both regions. This genotype has the worst prognosis and an adverse response to antiviral treatment.

In our study, HCV RNA could be detected in only 8.9% of serum samples from the borderline-positive cases as compared with 85% in the confirmed HCV antibody-positives. A diagnosis of HCV infection should never be based on a positive result in a screening assay alone.

In our study, the 26.3% of the healthy control population had already been HGV-infected. In comparison with the international prevalence data, this seems to be high. Transfusion or surgery before blood donor screening could be found in the case histories. HGV infection seems to occur in higher rates among HCV (50-62.5%), or hepatitis B virus (HBV)-infected patients (30%), but definite evidence linking it to a specific disease or illness is lacking.

When tenfold dilutions of two enterovirus strains, coxsackievirus B4 and echovirus 7, were tested by automated RNA extraction and real-time PCR on the LightCycler (LC) instrument, the detection limit was found to be 0.1 $TCID_{50}$ (50% tissue culture infective dose). When samples from the Third European Union Concerted Action Enterovirus Proficiency Panel were tested with automated RNA extraction and real-time PCR on the LC instrument and with the in-house assay, both molecular assays gave results identical to the expected

results, which were based upon the results of three reference laboratories using a total of four different molecular methods before distribution of the panel. From a total of 109 CSF specimens, 23 (21%) were repeatedly found to be positive by automated RNA extraction and real-time PCR on the LC instrument and by the in-house PCR assay, and 82 were found to be negative by both molecular assays. The new molecular assay allows rapid detection of enterovirus RNA in the CSF. It was found to be labor-saving and displayed sufficient sensitivity.

Abbreviations:

5'NTR	5' non-translated region
ALT	alanine aminotransferase
Anti-HCV	hepatitis C virus specific antibody
AST	aspartat aminotransferase
bDNA	branched DNA
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
EIA	enzyme immuno assay
ELISA	enzyme-linked immune assay
F	female
γ GT	gamma glutamil transferase
HAV	hepatitis A virus
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDV	hepatitis D virus
HEV	hepatitis E virus
HGV, GBV-C	hepatitis G virus (in this study only HGV will be used)
HIV	human immunodeficiency virus
IgG	immunoglobulin G
IgM	immunoglobulin M
IFN	interferon
IU	international unit
LC	LightCycler
LCR	ligase chain reaction
M	male
MEQ/ml	million genome equivalents/milliliter
N	negative
NASBA	nucleic acid sequence-based amplification
NAT	nucleic acid amplification technology
ND	not done
P	positive
PEG IFN	pegylated interferon
R	repeat result
RIBA	recombinant immunoblot assay
RB	ribavirin
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S/CO	sample rate/cutoff rate
SEN	variant of TTV detected first in Italy
TMA	transcription mediated amplification
TTV	transfusion transmitted virus
UNG	uracil-N-glycosylase (AmpErase®)
UV	ultraviolet

Preface

This thesis is based on results published or submitted for publication in the following papers, referred to in the text by their Roman numerals.

I. Müller Z., Deák J., Horányi M., Szekeres É., Nagy I., Ozsvár Z., Nagy E., Lonovics J., Gál G. The detection of hepatitis C virus in South Hungary. J Clin Virol 2001; 20: 81-83.
IF: 1,744

II. Müller Z., Deák J., Szekeres É., Berecki Cs., Nagy E., Túri S. A hepatitis C vírusfertőzés kimutatása gyermekkorban. Gyermekgyógyászat 2001; 52: 266-270. (in Hungarian)

III. Rabenau HF., Clarici A., Mühlbauer G., Berger A., Vince A., Muller Z., Daghofer E., Santner BI., Marth E., Kessler HH. Rapid detection of enterovirus infection by automated RNA extraction and real-time fluorescence PCR. J Clin Virol (in press)
IF: 1,744

IV. Müller Z., Deák J., Nagy E. Hepatitis G vírus – pathogén vagy endosymbionta? Klinikai Mikrobiológia és Infektológia (in Hungarian) (in press)

V. Müller Z., Deák J., Ross RS., Nagy E., Kovacs L., Kessler HH. Hepatitis C virus genotypes in Hungarian and Austrian patients with chronic hepatitis C. J Clin Virol (submitted to the journal)

VI. Deák J., Ujhelyi E., Tarján V., Szekeres É., Gál Gy., Brojnás J., Müller Zs., Lázár A., Weszelovszky E., Nagy K., Megyeri I., Tóth É., Nagy E. HIV és hepatitis vírusok szűrővizsgálatok eredménye a Szegedi Tudományegyetem Általános Orvostudományi karán dolgozók körében. Klinikai Mikrobiológia és Infektológia (in Hungarian) (in press)

1. Introduction

The profound influence of molecular techniques on all branches of biomedical science is most apparent in virology. Many newly identified viruses now known to cause significant diseases are diagnosed through the use of molecular techniques, even though they have not been characterized by the traditional methods of cell culture and infection of animal hosts.

Laboratory methods play a crucial role in establishing the differential diagnosis of hepatitis or enterovirus infections, monitoring the therapy and preventing the transmission of the disease, because the clinical signs and symptoms of the diseases are not always specific.

1.1. EXAMINATION OF HEPATITIS C VIRUS

Hepatitis viruses are challenging problems worldwide, despite the recognition of several viruses over the past few decades as being causative agents of this disease, and despite the development of methods for the detection of these viruses and of vaccines to prevent their spread.

The number of viruses that are known to cause hepatitis continues to increase. The artificial grouping of these totally unrelated viruses is based on the disease they cause: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), hepatitis G virus (HGV, GBV-C) or secondary hepatotrop viruses, such as several human herpesviruses, rubella, yellow fever, dengue and other hemorrhagic fever viruses [1]. The list of potential hepatotrop viruses continues to grow, with the recent discovery of the transfusion-transmitted virus (TTV) and SEN virus (SENV-D and SENV-H). In spite of numerous reports of the presence of these transmissible single-stranded, circular DNA viruses in various kinds of liver diseases with global distribution, definite evidence linking them to a specific disease or illness is lacking [2]. A putative hepatitis F virus has also been described and associated with a human stool sample; however, this awaits confirmation [3].

There are hepatitis viruses that are mainly transferred through the feco-oral route (HAV and HEV probably also including HDV and TTV), and the blood-borne viruses (HBV, HCV, HDV, HGV). The differential diagnosis of HCV and other hepatitis viruses is based on the case history data, clinical signs, biochemical and hematological laboratory findings and serological and molecular diagnostic methods. Blood, serum, plasma and liver tissue are

widely used for the investigation of these viruses, but stools, other tissues such as lymph nodes, saliva, semen and other excreta also can be investigated for the presence of viruses.

1.1.1. Features of HCV

The characteristics of HCV are summerized in Table 1.

Table 1. The features of hepatitis viruses

Virus	Hepatitis C
Family	Flavi
Genus	Hepacivirus
Genome	ssRNA (+)
Virion (nm)	30-60
Capsid	Yes
Antibodies	Anti-HCV, anti-HCV IgM
Transmission	Blood-borne, sexual, perinatal, unknown
Incubation (days)	14-180
Onset	Insidious
Chronicity rate	80-95%
Further disease	Hepatocellular carcinoma (HCC), cirrhosis
Mortality (%)	0.5-1
Active immunization	No
Therapy	Chronic: interferon (IFN)+ ribavirin (RB), pegylated IFN (PEG IFN)

1.1.2. HCV infection and its screening

According to the WHO data, it is estimated that 3% of the world population have been infected with HCV [(4)]. Therefore, there may be more than 170 million chronic carriers in the world 17% of whom are at risk of developing serious complications such as liver cirrhosis and HCC [(5)]. The prevalence of HCV infection varies from country to country; its rate ranges around 1% in Europe and the United States, between 1 and 3% in the Middle East and Asia, and between 1 and 19% in Africa.

Studies on HCV infections include the following populations: the screening of blood donors and risk populations such as post-transfusion patients, studies of community/acquired disease, studies of drug addicts, studies of hemophiliacs, common-source outbreaks and investigations of perinatal transmission.

The recent development of cell culture models for HCV has greatly advanced the study of HCV replication and the development of new therapies. At present, the most effective of these models is the replicon system [(6)].

Direct demonstration of the antigens of HCV is coming into the center of interest with the development of total HCV core antigen testing in the early phase of infection called the "window period", in spite of the low virus titer in the serum found to be 10^4-10^7 per ml in most patients [7].

The presence of the antibodies against HCV in human serum means that the infection has been acquired, and that the patient may carry the virus and can transmit it, but these antibodies do not help to eliminate the virus and their appearance does not mean protection against infection or superinfection. Antibodies against HCV antigens are present for years in both acute and chronic infections, but do not give information on the natural history.

Earlier, a high rate of false-positive enyzme immune assay (EIA) results was measured by a commercially available first-generation HCV EIA was described. The application of recombinant antigens in the second-(core, NS3 and NS4) and third- or fourth-generation (+NS5) tests increased both the sensitivity and the specificity. In the detection of HCV antibodies with EIA tests, we may be faced with indeterminate results. Several explanations can be found in these cases: falsely indeterminate results due to cross-reactivities with other viral antigens or immunological disorders, the "window period" at the onset of the infection, or a low virus titer in the serum, and a decreased host immunesystem reaction [8]. The "window period" decreased to 74 days following the appearance of second- and third generation EIA methods [9]. Second and third-generation recombinant immunoblot assays (RIBAs), where the individual antigen-antibody reaction can be read, may be used for confirmation. Second and third-generation assays have higher specificity and false-negative results are exceptional. These methods are suitable for the screening of blood donors or risk populations.

Patients found to be anti-HCV-positive should undergo HCV-RNA testing by molecular methods as part of the process of diagnosis [8].

1.1.2.1. HCV screening of blood donors

To prevent the nosocomial route of infection, the compulsory screening of blood donors was introduced in Hungary in 1992. The risk of HCV transmission, however, has been greatly reduced since the early 1980s by the selection of nonremunerated donors; donor selection to prevent viral transmission, initial surrogate testing in some regions, and the introduction of hepatitis B surface antigen (HBsAg), and HCV specific antibody (anti-HCV) testing have all contributed to this. Surrogate testing became obsolete following the introduction of virus-specific antibody and antigen screening.

The implementation of HCV antibody blood donor screening further decreased the transmission of the virus by blood transfusion in 1991-92 and the risk of post-transfusion hepatitis has declined from 1 in 5 000 to 1 in 200 000 [10].

1.1.2.2. HCV screening of health-care workers

Health-care workers are exposed to blood-borne infections to a greater extent than normal as a consequence of their work. By decision 18/1998. (VI. 3.) of the Minister of Social Welfare, health-care workers positive for human immunodeficiency virus (HIV), HBV or HCV must not participate in invasive medical treatment or dentistry, and screening is therefore necessary in this population.

Prospective studies have not been made; the risk of transmission from health-care workers was found to be 1/30 in the case of HBV, 1/300 in the case of HCV and 1/3 000 in the case of HIV calculated per surgical accident (Department of Epidemiology of B. Johan National Center for Epidemiology, unpublished). The Centre for Disease Control and Prevention (CDC) has not made specific recommendations, but the regulations of the insurance authorities against medical professional mistakes prevents virus carrier dentists, surgeons and gynecologists from continuing of their original specialized activities [4].

1.1.2.3. HCV infection of pregnant women and neonates

There is conflicting evidence on the rate of mother-to-infant transmission of HCV, which is explained by methodological differences. The transmission of HCV maz occur *in utero*, transplacentally at any time during pregnancy, during delivery or postnatally, and the relative importance of each of these routes remains unclear. The rate of perinatal transmission is approximately 6-7.8% in the case of HCV, but it is higher when the mother is coinfected with HIV [11, 12]. However, screening for HCV is not recommended for healthy pregnant women; the possibility of perinatal transmission cannot be prevented as in the case of HBV or HIV infection [13]. The route of perinatal transmission of HCV is not well understood, although several studies have been made worldwide. The identification of factors is essential to allow the development of effective prevention strategies in order to reduce the risk of perinatal transmission of HCV [14].

1.1.2.4. HCV infection among children

HCV infection is relatively infrequent among children (0.05-0.4%) as compared to the average adult European population (0.5-2%) [15]. Effective transmission routes such as

transfusion, hemodialysis, tattoo, piercing, needdlestick injury or intravenous drug use are rare or could not be found. There is not enough knowledge about the natural history, the clinical outcome and the efficacy of therapy of the disease in childhood. Before the implementation of HCV screening among blood donors, the most common route of HCV infection was parenteral. The mother-to-infant route subsequently took over the lead as we have no possibility to prevent such transmission. In countries where the prevalence of HCV is high among women of childbearing age, it can be an important transmission route, even with coinfection with HIV [16]. Children who have been recipients of multiple transfusions as a result of chronic anemia, cancer or hemophilia, and also as patients who have received extracorporeal membrane oxygenation or long-term parenteral nutrition, are at high risk of HCV infection; the reported prevalence ranges from 10 to 85% (Table 2) [17].

Table 2. HCV infection rate among high-risk pediatric populations

High-risk pediatric populations	Rate of HCV antibody positivity	Literature
Before 1992 blood or blood products		
Hemophilia or thalassemia	80%	Schwimmer et al. 2000[18]
Neonates for other reasons	2.5%	Grasselly et al. 1997[19]
Children recovered from malignant diseases	6.6%	Strickland et al. 2000[20]
Before 1992. open-heart surgery	14.6%	Vogt et al. 1999[17]
Before 1992. liver and heart transplantation	4-7%	Davidson et al. 1998[21]
Hemodialysis	1-54%	Pereira et al. 1997[22]
Mother-to-infant transmission	6-7.8%	Tajiri et al. 2001[12]

1.1.2.5. Chronic hepatitis C

Although 70-80% of HCV-infected persons become chronic carriers, most of them have a relatively mild form of the disease with slow progression [23].

Hepatitis is said to be chronic when the laboratory findings characteristic of hepatitis are above the normal level for at least 6 months. Clinical signs of chronic hepatitis are usually mild and nonspecific for a long time. The illness is often diagnosed in the late stage of liver disease. The onset of the infection therefore cannot be determined [1].

The histopathology encompasses the entire spectrum of liver damage ranging from acute fulminant hepatic necrosis to cirrhosis and HCC. Among those biopsied, about 20% display cirrhosis within 10 to 20 years, some of whom develop HCC [24].

The immune response to HCV appears to be critical both in determining viral clearance or persistence and in contributing to liver injury. Immunological investigations can be performed for examination of the host cellular and humoral immune response [25].

Differential diagnosis of other possible causes of chronic hepatitis (autoimmune hepatitis, other virus hepatitis, Wilson disease or hemochromatosis) and malignant diseases is important [26].

The natural history of HCV infection is not well understood because of the difficulties of setting up and maintaining suitable studies. Much of the data on the natural history has come from retrospective studies of people with chronic liver disease and is biased towards populations who experience the more severe consequences of the disease [5].

Treatment of patients with chronic hepatitis C according to international standards started in Hungary in 1992 [27]. The most effective and available initial therapy is currently combined treatment with IFN and RB, which results in a 35-40% sustained virologic response in all after a 24- or 48-week course of this therapy. Several circumstances influence the response to the therapy and many new drugs are under development [28].

Patients with chronic hepatitis C rarely have signs and symptoms for years, so this group is based on the screened population mentioned and those who already have signs or symptoms of hepatitis or other extrahepatic manifestations. Because of the possible occurrence of health-threatening diseases (cirrhosis and HCC) and careful selection for expensive therapy, these patients are followed up in one of the hepatology care units in Hungary [27].

1.1.3. HCV RNA detection

HCV replicates at relatively low levels and HCV RNA cannot be detected in body fluids by classical hybridization-based techniques; amplification is necessary [29].

There are a number of methods for qualitative HCV RNA detection: reverse transcription polymerase chain reaction (RT-PCR), transcription-mediated amplification (TMA), home-made RT-PCR, nucleic acid sequence-based amplification (NASBA), ligase chain reaction (LCR), real-time PCR and competitive RT-PCR-based assay (NGI). Many variations in the HCV PCR assay have been described in the literature, and standardization of such in-house assays has been difficult [30].

Numerous factors contribute to the variability of RT-PCR assays, including specimen handling and storage conditions, the presence of PCR inhibitors (5-10% of samples), the correct design of amplification primers, the variability of biochemical reactions, DNA product contamination, and the efficiency of post-amplification systems [31].

Occasionally, the results are borderline (defined in the case of Cobas Amplicor HCV Test version 2.0, Roche Diagnostics as an optical density of 0.15-1.0) and/or discordant upon repetition. These cases are indicative of very low-level viremia due either to an HCV infection or to transient contact with the virus, with or without subsequent HCV infection [32].

A standardized RT-PCR assay has been introduced, improved for clinical laboratories for the qualitative detection of HCV RNA in patient serum (Amplicor HCV 1.0, 2.0, COBAS Amplicor 2.0. Roche Molecular System, Pleasanton, California, USA). The detection limit of the latest assays reaches 50 International Units (IU)/ml. In newly described assays (Versant HCV RNA qualitative assay) the analytical sensitivity of 100% is 5 IU/ml [33]. The analytical sensitivity of assays is estimated according to WHO recommendations and is counted in IU instead of copies/ml and million genome equivalens (MEQ)/ml. If a result is positive, the diagnosis of ongoing HCV infection is confirmed.

The availability of effective antiviral therapy for hepatitis C has increased the need for the molecular detection and quantification of circulating hepatitis C viral particles. There are several methods for quantitative HCV RNA detection: RT-PCR, the bDNA method, NGI, TMA, real-time PCR, NASBA and LCR. Two of them are commercial, standardized assays and have been widely employed in the past few years in Hungary as well [34-36]. In the second-generation branched DNA-based signal amplification assay (Quantiplex HCV RNA 2.0, Chiron Diagnostics, Emeryville, California), the amount of luminescence emitted is proportional to the viral load, which is calculated in comparison with a standard curve established in each run with RNA standards in known amounts. A quantitative RT-PCR-based assay has also been developed and improved (Amplicor HCV Monitor 2.0, Cobas Amplicor 2.0, Roche Molecular Systems). The results of the assays are difficult to compare and this can lead to misinterpretation of the results [8].

1.1.3.1. HCV RNA detection in the diagnosis of HCV infection among blood donors, health-care workers, pregnant women, neonates, children and patients with chronic hepatitis C

The introduction of HCV nucleic acid amplification technology (NAT) as a quality control of manufacturing pools for plasma products or as a form of blood donor screening by

minipools is anticipated in many European countries [37]. The test detects HCV RNA 23 to 32 days prior to seroconversion [38]. The introduction of highly automated NAT (Ampliprep, Roche) and microarray systems and multiple PCR helps to resolve the problem of mass screening and diagnostics [39]

Because of the diagnostic mix of HCV infection, it is difficult to investigate the prevalence and natural history of infection of a population throughout the world. In the event of a naturally immunosuppressed status such as pregnancy, the diagnosis should be based on the detection of HCV RNA [40].

The diagnosis of infection in babies has some unique characteristics. Although it is possible to detect HCV RNA immediately after birth, it is prudent to delay the diagnosis of perinatal transmission. The diagnosis of perinatal transmission should be based on the presence of HCV RNA in at least two serum samples after the second month postpartum, while anti-HCV should be confirmed after the first year of life, since maternal anti-HCV antibodies may persist 20 months after birth. After infancy, a variable percentage of perinatally infected children are anti-HCV-negative with detectable HCV RNA in the serum. Thus, HCV RNA should be determined in all children to confirm the diagnosis [14]. According to the literature, elective cesarean section can reduce the risk of perinatal transmission; in this way, transmission could be prevented [40].

In cases of chronic hepatitis C, pretreatment HCV viral load and the rate of virus decline during therapy have been shown to be helpful in making recommendations regarding treatment [41].

1.1.4. HCV genotype detection and phylogenetic analysis

The molecular characterization of HCV revealed the existence of a positive-sense RNA genome approximately 9400 bases in length. The complete genome sequence has been determined in different HCV isolates worldwide and substantial nucleotide sequence variability along the complete viral genome has been found [42]. The comparison of the published sequences of HCV has led to the identification of distinct HCV genotypes that may differ from each other by as much as 33% over the entire viral genome [43]. The variability within the HCV genome has formed the basis for several genotyping systems. The currently most commonly used classification system is based on the 5′ nontranslated region (5'NTR) [44]. HCV genotypes 1, 2 and 3 exhibit a worldwide distribution, while others, such as types 5 and 6, are found only in specific geographical regions [45]. The different genotypes are

relevant to epidemiological questions, vaccine development, and the clinical management of chronic HCV infection.

Methods for HCV genotyping include the amplification of certain genomic regions, followed by nucleotide sequencing, RT-PCR with genotype-specific primers, restriction fragment length polymorphism (RFLP) analysis of RT-PCR products and RT-PCR with universal primers followed by subtype-specific hybridization [46-49]. Nucleotide sequencing followed by phylogenetic analysis is the current "gold standard" for identifying different HCV genotypes and subtypes, but is generally regarded as impractical for routine clinical laboratory settings [50]. Recently, the TruGene HCV 5'NC Genotyping Kit (Visible Genetics, Toronto, Ontario) has come onto the market. For this assay, amplification products generated by the AMPLICOR HCV or AMPLICOR HCV MONITOR assays (Roche Molecular Systems, Pleasanton, CA) can be used.

1.1.4.1. HCV genotype detection and phylogenetic analysis in the diagnosis and management of HCV infection among blood donors, health-care workers, pregnant women, neonates, children and patients with chronic hepatitis C

With the exception of intravenous drug users for whom a characteristic distribution of viral types has been convincingly and reproducibly shown, the extensive search for preferential genotype segregation within the various patient categories has by and large been unsuccessful. The genotype distribution varies with the age of the patients, as a likely consequence of temporally distinct epidemic waves [51].

Molecular biology can be used to furnish evidence of nosocomial transmission and for the documentation of hemodialysis patient-to-patient transmission in hemodialysis units or in women originally infected through exposure to a single batch of HCV-contaminated anti-D immunglobulin [52].

The influence of the viral genotype in the pathogenesis of liver disease is still controversial, although genotype 1 is associated with a poor response to treatment with IFN alone or in combination with RB [53].

1.2. EXAMINATION OF HEPATITIS G VIRUS

Newly discovered blood-borne hepatitis viruses were found through the investigation of transfusion-associated non-A, non-B hepatitis agents. In 1995, GB virus C (GBV-C) [54] or

HGV [55] was identified in two laboratories, as independent representatives of the same virus. The term HGV will be used in this thesis.

1.2.1. Features of HGV

HGV has a positive-sense RNA and belongs in the family of *Flaviviridae.* The genome of HGV is similar to the HCV genome in 5'NTR. Because of a relatively low level of amino acid sequence variation among the strains, HGV is classified into groups rather than types and subtypes. According to Smith et al. [56], there are four groups (1, 2, 3 and 4) and two subgroups in group 2 (subgroups 2a and 2b).

Transmission via the blood-borne route is the most common mode, although perinatal and sexual transmission are well documented [57, 58].

1.2.2. Diagnosis of HGV infection

The diagnosis of acute HGV infection is based on RT-PCR [59] as there are no serological assays to detect current HGV infection. In cases of past infection, EIAs for the detection of antibodies to the E2 protein (anti-E2) of HGV have been established by several laboratories [60]. Interestingly, almost all sera positive for anti-E2 were negative for HGV RNA. This means that anti-E2 correlates with HGV clearance. Thus, anti-E2 positivity means past, recovered HGV infection. This suggests that tests for HGV RNA and anti-E2 are very useful to assess the rate of infection by this virus.

1.2.3. Prevalence of HGV infection

The prevalence of HCV RNA in healthy blood donors has been reported from around the world. Though HGV infection is common worldwide, the prevalence ranges from 0.6 to 14.2% [61]. In Hungary the prevalence has been examined among 60-year-old and older, healthy persons (28%), and healthy health-care workers (26%) [62, 63]. In high risk groups, such as persons with involved in intravenous drug use or hemodialysis the prevalence can be 19-78% [64].

1.2.4. Role of HGV infection in chronic hepatitis

The virus can provoke both acute and chronic infection and appears to be sensitive to IFN. As the route of infection seems to be similar to those for HBV and HCV infections, coinfection with HBV and/or HCV is common (15%) [65]. The role of HGV in chronic hepatitis remains uncertain [66].

1.3. EXAMINATION OF ENTEROVIRUSES

1.3.1. General introduction

The enteroviruses comprise a genus of the family *Picornaviridae* [67]. The spread of the virus is primarily via the oral-fecal route, perhaps with some respiratory involvement. Transmission through blood or blood products is possible [68]. While many enterovirus infections result in no clinical consequences or only mild illness, members of this group of viruses can cause many different diseases, occasionally affecting a variety of target organs: neurologic (flaccid paralysis, aseptic meningitis or encephalitis), respiratory (common cold, tonsillitis, pharyngitis, rhinitis, pleurodynia, hand-feet-mouth disease or conjunctivitis) or cardiovascular (myocarditis or pericarditis), but almost all members can cause aseptic meningitis [69]. In addition to the known and documented enteroviral infections, these viruses have also been implicated (but definitive proof is lacking) in several chronic diseases, some of which are: juvenile onset of diabetes mellitus, chronic fatigue syndrome, amyotrophic lateral sclerosis (Lou Gehrig's Disease) and Guillain-Barré and postpolio syndromes [70]. Nonbacterial 'aseptic' meningitis is a common manifestation of enterovirus infection.

Table 3. Enterovirus types based on 5'NTR sequences [71-73]

Clusters of Enterovirus genus	Viruses	Major disease
"Poliovirus-like"	Polioviruses 1-3	Flaccid paralysis, **aseptic meningitis**
	Coxsackieviruses A1-21, 24	**Aseptic meningitis** herpangina, conjunctivitis (A24)
	Enterovirus 70	Conjunctivitis
"Coxsackievirus B-like"	Coxsackieviruses B1-6	**Aseptic meningitis,** encephalitis, fatal neonatal disease, myo- or pericarditis, pleurodynia (Bornholm disease)
	Coxsackieviruses A9, 16,	**Aseptic meningitis,** human hand-feet-mouth disease (A16)
	Echoviruses 1-9, 11-27, 29-34	**Aseptic meningitis,** rashes, febrile illness, myocarditis
	Enteroviruses 68, 69, 71	**Aseptic meningitis,** polio-like illness, human hand-feet-mouth disease (enterovirus 71)
Parechovirus	Enteroviruses 22-23	**Aseptic meningitis,** encephalitis
Kubovirus	Aichi virus	**Human enterocolitis**
Hepatitis A cluster	HAV and certain CAV	**Hepatitis epidemica**

* Echovirus 9 = coxsackievirus A23; echovirus 10 = reovirus; echovirus 28 = rhinovirus; echovirus 34 = coxsackievirus A24. In the current report of the International Committee on Taxonomy of Viruses, these viruses are prefixed 'human' to distinguish them from animal viruses where relevant.

1.3.2. Diagnosis of enterovirus infection

The usual specimens for virus isolation are feces (or rectal swabs) and throat swabs. Culture or nucleic acid detection of virus in the cerebrospinal fluid (CSF) is an essential part of the routine laboratory diagnosis of patients with aseptic meningitis. The laboratory diagnosis of enterovirus meningitis has concentrated on isolation of the virus from CSF. Attempts to isolate enteroviruses, however, are laborious, time-consuming, and frequently unsuccessful [74-76].

1.3.2.1. Enterovirus RNA detection

Molecular assays for the detection of enteroviruses have been introduced and shown to be quicker and more sensitive than cell culture methods [77-81]. However, conventional in-house molecular assays based on RT-PCR require special equipment and staff and are not suitable for a high-throughput routine diagnostic laboratory. Furthermore, they lack standardization. Therefore, a more standardized and easier-to-handle molecular assay, the AMPLICOR Enterovirus Test, has been introduced and shown to be suitable for the routine diagnostic laboratory [79, 82]. This assay, however, has recently been removed from the market.

1.3.2.2. Automated specimen preparation and real-time PCR

The MagNA Pure LC (Roche Molecular Biochemicals, Mannheim, Germany), a fully automated specimen preparation instrument, and the LightCyler (LC) instrument (Roche), which allows high-speed thermal cycling and online real-time fluorescence monitoring, have recently been introduced [83, 84]. Combined use of these instruments allows the establishment of rapid molecular assays for the detection of pathogens in the routine diagnostic laboratory.

1.3.3. Role of enterovirus infection among pregnant women, neonates and children

Pregnancy and parturition may be associated with a greater risk of myocarditis with coxsackie B infection. Maternal antibody may not affort protection from infection, but will prevent clinical disease [85].

A kind of enterovirus seroconversion occurs in 60% of pregnancies; in 25% of pregnancies, there are two or more kinds of enterovirus seroconversion. Prematurity, low birth weight, onset of illness within the first few days of life, occurrence during the 'enterovirus season' and recent antepartum or postpartum febrile illness in the mother are risk factors for the most severe, generalized and fatal neonatal enteroviral disease, most commonly associated

with the coxsackie B virus, particularly B2 and B4. In temperate climates, enteroviruses are more prevalent in the summer and autumn (i.e. when ancient temperature and humidity are at their peak). The frequency of enterovirus infections amongst neonates is in 0.6-0.7%. Neonatal illness has seldom been attributed to coxsackie A viruses (because of their difficult diagnostics), but echoviruses (e.g. types 4, 9, 11, 17-20, 22 and 31) have been incriminated in both nursery outbreaks and sporadic infections [86]. Fatal cases of echovirus infection generally present with progressive severe hepatitis, myocarditis and CNS involvement, whereas fulminant coxsackievirus B infections can be distinguished by the presence of severe myocarditis and serious meningitis.

Young children in a community are the usual reservoir of enterovirus infection. In young infants, enteroviral meningitis is often confused with illnesses caused by bacteria and herpesvirus, resulting in thousands of children being hospitalized and incorrectly treated with antibiotics and anti-herpes drugs. Rapid and correct diagnosis could prevent such unnecessary treatment [87]. It is essential to establish a diagnosis as quickly as possible in order to institute measures to prevent infection spreading to other infants.

2. Aims of the study

This study was carried out with the following aims:

2.1. EXAMINATION OF HEPATITIS C VIRUS

- To investigate the epidemiology of HCV infection among healthy (blood donors and pregnant women) and risk populations (health-care workers and children who received blood or blood products before the imlementation of blood donor screening) in South Hungary.
- To compare the usefulness of EIA and RT-PCR methods for the detection HCV infection
- To determine the HCV genotypes among patients with chronic hepatitis C in South Hungarian by automated nucleotide sequencing of HCV 5'NC and phylogenetic analysis.
- To analyze data in comparison with those obtained from a randomized number of patients with chronic hepatitis C in South-east Austria.

2.2. EXAMINATION OF HEPATITIS G VIRUS

- To determine the anti-E2 prevalence in high-risk groups, such as chronic HCV-infected patients and health-care workers.

2.3. EXAMINATION OF ENTEROVIRUSES

- To evaluate a qualitative molecular assay based on automated RNA extraction on the MagNA Pure LC instrument and real-time PCR with the LC system.
- To investigate clinical specimens after determination of the detection limit.
- To assess the sensitivity of a qualitative in-house PCR assay.

3. Patients and Methods

3.1. POPULATIONS STUDIED FOR HEPATITIS C AND G VIRUSES

3.1.1. Blood donors

From 1992 onwards a total of 45 719 blood donors were examined for the presence of anti-HCV antibodies. The median age of the blood donors was 35 years (range: 19 to 74 years). All confirmed anti-HCV-positives were interviewed for potential risk factors of HCV transmission and tested with RT-PCR for the presence of HCV RNA and serum viral load. RT-PCR-positives were tested for genotype. The resulting data were compared with those obtained on a randomized number of anti-HCV-negatives.

3.1.2. Health-care workers

Between May 1999 and January 2000 serum samples from 2051 health-care workers (female/male: 1130/921) were examined for the presence of HBsAg and anti-HCV. The median age of the male health-care workers was 42.49 years (scatter: 13.92), and that of the females 38.04 years (scatter: 11.59). In the HBsAg and anti-HCV-positive workers further examinations were carried out (see below). In the cases of 94 health-care workers (female/male: 51/43, median age: 47 years), anti-HGV examinations were also performed. These health-care workers were interviewed for potential risk factors of HBV and HCV transmission.

3.1.3. Pregnant women

Serum samples from 500 healthy pregnant women were examined for the presence of HBsAg and anti-HCV for the possibility of perinatal transmission of HBV and HCV. The mean age of the pregnant women tested was 26 years (range: 17 to 38 years). All confirmed anti-HCV-positives and HBsAg-positives were examined further.

3.1.4. Children

A total of 120 children (female/male: 61/59) who had received one or more blood transfusions in their early childhood prior to implementation of anti-HCV blood donor screening were tested for anti-HCV positivity and their liver functions were tested by measurement of the serum GPT level. The mean age of these children was 7 years (range: 5 to 10 years). The resulting data were compared with those obtained from a randomized number of children not belonging in HCV risk groups. All those children who gave positive anti-HCV results were tested further (RT-PCR, genotype and HGV anti-E2).

3.1.5. Patients with chronic hepatitis C

A group of 126 patients (female/male: 54/72) with the diagnosis of chronic hepatitis C were followed up during treatment (IFN and IFN+RB) for the presence of anti-HCV and HCV RNA. Between 1992 and 2001, these patients received antiviral therapy. The median age of the patients with chronic hepatitis C was 46 years (range: 15 to 73 years). In 48 patients with chronic hepatitis C (female/male: 20/28, average age: 46.5 years), anti-HGV detection was performed. In 18 cases, HCV genotype detection was also carried out.

Borderline reactive anti-HCV samples were examined in 79 individuals (female/male: 47/32, median age: 40 (0.6-77) years) and HCV RT-PCR was performed in 50 persons (female/male: 35/15, median age: 39 (8-70) years) despite the negative serology.

HGV examinations were made in the cases of 48 patients with chronic hepatitis C (female/male: 20/28, average age: 46.5 years) and 94 health-care workers (female/male: 51/43, median age: 47 years). Case history and physical examinations were carried out at the hepatologic outpatient clinic.

3.2. METHODS FOR STUDY OF HEPATITIS C AND G VIRUSES

3.2.1. Collection of specimens

Within 3 hours following blood draw, blood collection tubes were centrifuged and serum aliquots were prepared and were either tested immediately or frozen at –80 °C until testing. All patients were sent to the Hepatology Centre, at the 1st Deparment of Internal Medicine, University of Szeged for follow-up. Liver function was screened by serum GPT level using UV, IFCC method on a Hitachi 917 automata at the Department of Clinical Chemistry, University of Szeged, according to the manufacturers instructions. Liver biopsy was done in the case of patients with chronic C virus hepatitis.

3.2.2. Serological assays

All sera were screened for the presence of anti-HCV by either second- or third-generation EIAs (Abbott Axsym EIA 2.0, 3.0). Positive results were confirmed by either second- or third-generation recombinant immunoblot assays (Ortho RIBA 2.0, 3.0). According to the recommendation of the German Federal Republic Authority (Paul-Erlich-Institute, Langen, Germany) the borderline reactivity of Abbott Axsym EIA tests is sample/cutoff ratio (S/CO): 0.80-0.99. In our study the borderline reactivity was taken as S/CO: 0.8-1.5.

HBsAg was tested using the ELISA Test System 3 (Ortho, Budapest) or Axsym V2 (Abbott, Szeged). Confirmations were performed by using the Hepanostica Uniform HBsAg Test 2.0 or the Confirmatory Test 2.0 (Organon, Budapest). In the cases of reactive samples,

other hepatitis B virus markers were detected by the Hepanostica Uniform anti-HBc IgM, anti-HBc IgG, anti-HBe, HBeAg Elisa Test (Organon, Budapest).

HGV antibody E2 was detected by the RD System anti-GBV-C test. The assays were performed according to the instructions of the manufacturers.

3.2.3. Qualitative and quantitative HCV RNA detection

For qualitative detection of HCV RNA in the serum samples, either the Amplicor HCV Test 2.0 or the Cobas Amplicor HCV Test 2.0 (Roche Molecular Systems, Pleasanton, California, USA) was used. This assay is carried out in a semi-automated manner, including manual extraction and automated amplification, in the case of Cobas automated PCR product hybridization and interpretation. The use of an internal control excludes the possibility of false-negative results. The risk of false-positive results can be lowered by careful hands-on work and with the use of uracil-N-glycosylase (UNG). The specificity of the Amplicor HCV 2.0 appears to be of 97-99%. The detection limit is 50 IU/ml.

For quantitative detection of HCV RNA the bDNA technique (Quantiplex HCV RNA 2.0, Chiron Diagnostics, Emeryville, California) was applied at the Viral Nucleic Acid Laboratory, National Institute of Hematology and Immunology. The method is based on the fact that the amount of luminescence emitted is proportional to the viral load, which is calculated in comparison with a standard curve established in each run with RNA standards in known amounts. The viral load is given in MEQ/ml or converted into IU/ml.

3.2.4. Genotype detection and phylogenetic analysis of HCV

For the sequencing of 5'NTR of HCV by Trugene kit the amplified products of RT-PCR performed by COBAS AMPLICOR kit (Roche) are required. After amplification and denaturation, the amplified products has to be used immediately or frozen down for one week for further examination. Before sequencing, the PCR products were neutralized and purified by DNA-binding resin system (High Pure PCR Product Purification Kit, Boehringer Mannheim, Germany). All samples were sequenced by a bidirectional DNA sequencing method (CLIP, Visible Genetics, Toronto, Canada). A set of 4 reactions was prepared containing standard dideoxynucleotide termination reagents and two different far-red fluorescent dyes linked to the two inward–facing oligonucleotide primers. One of the primers was labeled with Cy5.0 and the other with Cy5.5. The PCR was performed in a single run for 45 cycles generating chain-terminated reaction products from each primer. The automated sequencing of the 183-bp fragment was performed in 4 lanes of an ultrathin (50-μm-thick) disposable polyacrylamide gel on a MicroGene Clipper sequencer.

The unique feature of this method is to provide sequence information for both positive and negative DNA strands in a single reaction.

"GeneObjects" software was used to analyze the chromatograms of each sample: the final 5' NTR sequence was obtained from the comparison of both complementary strands. This information was compared with deposited HCV sequences by "Gene librarian TM" software with a minimal concordance cut-off of 98%.

The sequencing system is also able to detect mixed populations if a single strain represents at least 10-15% of the total amount.

Sequences of the 183-bp HCV 5'NCR (nt 96 to 278) DNA fragments from all samples were subjected to phylogenetic analysis, using the PHYLIP software package, version 3.5c [50]. Distances between pairs of sequences were estimated with the DNA-DIST program. Phylogenetic trees were constructed by the unweighted pair group method using arithmetic averages on the previous sets of pairwise distance.

3.2.5. Statistical analysis

Data were analyzed with the two-sided Fisher's exact test and the Mann-Whitney U test.

3.3. STUDY DESIGN OF ENTEROVIRUS INFECTION FROM CSF SAMPLES

A total of 109 CSF specimens were investigated for the comparative study. First, two enterovirus strains, coxsackievirus B4 (VR-184) and echovirus 7 (VR-37) from the American Type Culture Collection (Rockville, Md.) were used for determination of the detection limit of the qualitative molecular assay based on automated RNA extraction and real-time PCR. Enterovirus RNA was extracted from tenfold dilutions of viral stocks ranging from 1000 to 0.01 $TCID_{50}$ (50% tissue culture infective dose). Experiments were repeated five times on different days.

In the second step, the Third European Union Concerted Action Enterovirus Proficiency Panel, which contained different concentrations of coxsackievirus A9, coxsackievirus B5, echovirus 6, echovirus 11 and enterovirus 71 was used (Table 4). Samples were blind tested by both, the new qualitative molecular assay based on automated RNA extraction and real-time PCR and the in-house molecular assay.

In the third step, a total of 109 CSF specimens were investigated in a comparative study. Hungarian CSF specimens had been collected from patients admitted to various departments of the University of Szeged (Hungary). All patients had signs and laboratory findings of meningitis.

Table 4. Third European Union Concerted Action Enterovirus Proficiency Panel

Vial number	Virus	Dilution[a]	Expected result[b]
1	Coxsackievirus A9	10^{-8}	negative
2	Coxsackievirus A9	10^{-7}	positive
3	Coxsackievirus A9	10^{-6}	positive
4	None		negative
5	Echovirus 6	10^{-4}	positive
6	Echovirus 11	10^{-6}	positive
7	Coxsackievirus B5	10^{-5}	positive
8	Enterovirus 71	10^{-5}	positive
9	Echovirus 11	10^{-3}	positive
10	None		negative
11	Echovirus 11	10^{-5}	positive

[a] Dilution of original stock; $TCID_{50}$/ml of original virus stock; coxsackievirus A9, 3.6×10^6; coxsackievirus B5, 3.2×10^7; echovirus 6, 2.0×10^8; echovirus 11, 2.5×10^7; enterovirus 71, 5.6×10^6.

[b] Expected results are based upon the results of three reference laboratories using a total of four different molecular methods before distribution of the panel.

3.4. METHODS FOR THE DETECTION OF ENTEROVIRUSES

3.4.1. Collection of specimens

All specimens had been obtained prior to the start of therapy. Immediately after lumbar puncture, aliquots were prepared and frozen at –70°C. Aliquots were labeled using double blind system and sent for investigation with the new molecular assay to the Institute of Hygiene, Graz, Austria, and for investigation with the in-house molecular assay to the Institute of Medical Virology, Frankfurt am Main, Germany. Each sample was tested twice with the new molecular assay. Each positive result was confirmed by a second identical PCR run.

3.4.2. RNA extraction

For the new molecular assay, enterovirus RNA was extracted with the Total Nucleic Acid Isolation Kit (Roche) on the automated MagNA Pure LC instrument. The extraction protocol "Total Nucleic Acids Serum, Plasma, Blood" was employed. A specimen volume of 200 µl was used. An elution volume of 100 µl, and a dilution volume of 0 µl were chosen. Other details, such as reagent volumes and the number of reaction tips needed for the run, were automatically calculated by the software. The MagNA Pure LC automatically performed all the remaining steps of the procedure with specially designed nuclease-free, disposable reaction tips. These reaction tips transfered the samples and also served as reaction vials for the procedure. Within the tips, nucleic acids were bound to magnetic beads, washed free of impurities, and finally eluted from the magnetic beads into a cooled sample cartridge. Reaction tips after use were automatically discarded during the run into an attached, autoclavable waste bag.

After the completion of DNA extraction, the MagNA Pure LC Cooling Block, which included the sample carousel with an adequate number of LC capillaries, and the reaction vessel including the master mix were placed into the postelution area. After the start of the postelution protocol, which had been programmed prior to the start of the first run, the MagNA Pure LC automatically pipetted 16 µl of the master mix and 4 µl of the processed sample into each of the LC capillaries.

For the in-house molecular assay, enterovirus RNA was isolated from 200 µl of CSF with the commercially available QIAamp HCV Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations.

3.4.3. Primer design

For the seminested real-time PCR assay, oligonucleotides deduced from the highly conserved 5'-nontranslated region of the enterovirus genome were used. The outer primers, which were identical to those used for the former AMPLICOR Enterovirus Test, allowed amplification of a 149-bp fragment; the final fragment, obtained after completion of the second round, had a length of 85 bp. The TaqMan probe (TIB MOLBIOL, Berlin, Germany) was labeled with 6FAM at the 5'end and with TAMRA at the 3'end. The primer and probe sequences and characteristics are shown in Table 5.

For the in-house PCR assay, oligonucleotides were derived from the highly conserved 5'-noncoding region as reported earlier [88, 89]. The product, obtained after single-round PCR, had a length of 287 bp.

3.4.4. Real-time PCR on LC instrument

The real-time PCR was performed on the specially designed LC instrument. Evaluation of the different assay formats has been described in detail elsewhere [84]. First-round LC-PCR was performed with the LightCycler RNA Master Hybridization Probes kit (Mn^{2+} final concentration, 5.5 mM; primer final concentration, 0.5 µM each). Sixteen microliters of master mix and 4 µl of RNA template were automatically pipetted into each glass capillary by the MagNA Pure LC. Sealed capillaries were centrifuged in a microcentrifuge and placed into the LC rotor. Reverse transcription was performed for 20 min at 61°C. After denaturation for 2 min at 95°C, 60 LC-PCR cycles were run. After this, another denaturation was carried out for 5 seconds at 95°C. For the 10-s annealing, a touch-down profile (70° to 62°C; step size, 0.2°C per cycle) was employed. Elongation was done for 10 s at 72°C. After the first-round LC-PCR, capillaries were opened and the reaction mixes were centrifuged into 1.5-ml tubes. After this, 980 µl of bidistilled water was added into each tube. For the second round, 2 µl aliquots of these dilutions were added to 18 µl of LightCycler Fast Start DNA Master

Hybridization Probes kit (Mg^{2+} final concentration, 4 mM; primer final concentration, 0.5 µM each). After denaturation for 10 min at 95°C, 30 LC-PCR cycles were run. Another denaturation followed for 5 s at 95°C. For the 10-s annealing, another touch-down profile (65 to 60°C; step size, 0.2°C per cycle) was employed. An elongation step was finally carried out for 10 s at 72°C.

3.4.5. In-house RT-PCR and detection of amplification products

Enterovirus RNA was eluted with 50 µl of preheated (95° C) bidistilled and DEPC-treated water and 20 µl were taken for reverse transcription and amplification using the Qiagen OneStep RT-PCR Kit (Qiagen) according to the manufacturer's instructions (final concentration of primers, 0.5 µM each). Reverse transcription (RT) was carried out at 50° C for 30 min. After stopping the reaction at 95° C for 15 min, amplification of cDNA was carried out in the same tube. Amplification involved 35 cycles on a Perkin-Elmer 9600 thermocycler. Thirty-five PCR cycles consisting of 30 s at 94° C, 30 s at 50° C, and 30 s at 72° C were run. After the final cycle, the tubes were incubated for an additional 5 min at 72° C. Each amplification run contained three negative controls and one positive control. Amplification products were analyzed by gel electrophoresis on a 2% agarose gel. All positive results were confirmed by a complete second run.

Table 5. Oligonucleotides used for the new molecular assay

Assay step and primer (sequence) or product	Positions amplified[a]	Length (nucleotides)	G+C content (%)	Melting temp (°C)
Initial PCR				
Primers				
Forward (5′-CCCCTGAATGCGGCTAATCC)	455-474	20	60.0	61.7
Reverse (5′-CAATTGTCACCATAAGCAGCCA)	582-603	22	45.5	58.8
Product	455-603	149		
Seminested PCR				
Primers	517-536	20	60.0	60.2
Forward (5′-GTAACGGGCAACTCTGCAGC)	582-601	20	45.0	55.2
Reverse (5′-ATTGTCACCATAAGCAGCCA)				
Product	517-601	85		
TaqMan probe (5′-6FAM-CACGGACACCCAAAGTAGTCGGTTCC-TAMRA)	537-562	26	55.6	69.0

[a]GenBank accession number: AF231765 (Coxsackievirus B3 strain PD, complete genome).

4. Results

4.1. EXAMINATION OF HEPATITIS C VIRUS

HCV infection was examined in SouthHungary among blood donors, health-care workers, pregnant women, newborns, children and patients with chronic hepatitis C with known case history data, ALT and anti-HCV findings, in addition to the qualitative RT-PCR, quantitative bDNA and in 20 cases genotype identification based on the 5'NTR nucleotide sequence. For 19 viruses, phylogenetic analysis was also possible. The results are summarized in Table 6.

Table 6. HCV infection screening in different population

Laboratory findings	Number of blood donors tested: 45719	Number of health-care workers tested: 2051	Number of pregnant women tested: 500	Number of neonates tested born to HCV infected mothers tested: 7	Number of healthy children tested (control group): 50	Number of children tested who received blood or blood products before 1992 tested: 120	Number of chronic hepatitis C patients tested: 126
Mean ALT values (U/l)	54	*	45	15	40	79,5	94
Number of confirmed anti-HCV-positive persons (%)	45 719/195 (0.42%)	2 051/11 (0.53%)	500/5 (1%)	7→0** (0%)	50/0 (0%)	120/2 (1.7%)	126/126 (100%)
RT-PCR in anti-HCV-positive persons (%)	47/40 (85%)	11/10 (90%)	5/3 (60%)	0 (0%)	0/0 (0%)	2/2 (1.7%)	126/126 (100%)

- * not tested
- ** transient postnatal transplacental anti-HCV positivity

4.1.1. Blood donors

Altogether 195 (0.42%) of the 45 719 blood donors were confirmed anti-HCV-positives. The female/male ratio was 65/130. All 195 confirmed anti-HCV-positive blood donors and 390 randomised anti-HCV negative blood donors were interviewed for potential risk factors of HCV infection. In 408 patients, one or more risk factors could be identified, whereas in the case of 154 persons, no risk factors were found (Table 7).

Table 7. Risk factors of anti-HCV-positive and anti-HCV negative blood donors

Risk factor	Confirmed anti-HCV-positive (*n*=195)			Anti-HCV-negative (*n*=390)			***P*** **(total)**	**Odds ratio**
	M[a] (*n*=130)	F[a] (*n*=65)	Total (%)	M (*n*=220)	F (*n*=170)	Total (%)		
Surgery	61	57	118 (60.5)	54	84	138 (35.4)	**<0.0001**	**2.798**
Transfusion	50	28	78 (40.0)	41	42	83 (21.3)	**<0.0001**	**2.466**
Insertion of earrings	9	64	73 (37.4)	4	150	154 (39.5)	**0.654***	
Tattooing	39	3	42 (21.5)	18	2	20 (5.1)	**<0.0001**	**5.078**
Number of pregnancies >3	0	19	19 (9.7)	0	18	18 (4.6)	**0.001**	**3.488**
Acupuncture	4	5	9 (4.6)	4	9	13 (3.3)	**0.491**	
Health-care worker	3	5	8 (4.1)	4	6	10 (2.6)	**0.318**	
Intravenous drug abuse	2	0	2 (1.0)	0	0	0	**0.111**	
Multiple risk factors	**105**	**64**	**169 (86.7)****	**84**	**153**	**237 (60.7)**	**<0.0001**	**4.196**
No risk factors	**25**	**1**	**26 (13.3)**	**136**	**17**	**153 (39.2)**		

[a] Abbreviations: F, female; M, male

* $P < 0.05$ in subgroup of females

** $P < 0.0001$ compared to patients without risk factors

The difference was significant by sex (*P* male: 0.019, OD: 4.017; *P* female: 0.011, OD: 8.533)

Comparison of the groups revealed that those who had undergone surgery, transfusion, tattooing, the insertion of earrings by sex or more than three pregnancies were significantly more at risk of acquiring HCV infection. Although the rate of health-care staff was twice as high in the anti-HCV-positive group, the difference was not significant. No significant difference could be found in the cases of intravenous drug use or acupuncture. Those who had multiple risk factors had more than a four times higher risk of aquiring HCV infection.

HCV RT-PCR was performed in 47 of the anti-HCV-positive blood donors. In 40 of them (85%), serum HCV RNA was detected, while in the remaining 7 blood donors (15%), the HCV PCR gave a negative result. Those who were shown to be HCV carriers were sent to the Hepatology Care Unit for further follow-up examinations and therapy.

4.1.2. Health-care workers

From 2 051 serum samples screened for the presence of HCV antibody and risk factors in the anamnesis, 31 were positive and 5 were borderline-positive These HCV antibody positive persons were retested simultaneously with a second HCV ELISA and PCR methods. The 5 borderline-positive individuals proved to be negative with both of the alternative procedures. Only 11 of the 31 originally HCV-positive persons were found to be positive with the second ELISA and 10 of them also proved to be virus carriers. The HCV serology was negative with the second ELISA in 3 patients, but the PCR indicated a virus carrier state.

4.1.3. Pregnant women and neonates

Five persons (1%) from among the pregnant population screened were found to be anti-HCV-positive and 3 of them (0.6%) were also HCV carriers. The serum ALT level was elevated in one person. All in all, 7 babies were examined for perinatal transmission of the virus. Two of them had not been included in the screened population. After a transient postnatal anti-HCV positivity, none of the children became or remained anti-HCV or HCV RNA-positive later during follow-up (time schedule: 1 month after birth: anti-HCV, RT-PCR; 12 months: anti-HCV, RT-PCR; 24 months: anti-HCV, RT-PCR).

4.1.4. Children

Two (1.7%) of 120 children who had received blood transfusions prior to the anti-HCV screening, were confirmed to be anti-HCV-positives. Both of them had genotype 1b HCV RNA. Their serum ALT levels were persistently elevated (mean ALT: 79.5 U/l). The serum HCV RNA level of one of them remained below the limit of detection with the bDNA technique. The other patient had 0.644 MEQ/ml in the serum.

4.1.5. Patients with chronic hepatitis C

Patients found to be HCV-infected were followed up. All of them (126) were positive for both anti-HCV and HCV RNA at the beginning of the follow-up. The mean ALT value was 94 U/l (range: 51 to 265 U/l).

The median serum HCV RNA load was found to be 7.6×10^5 IU/ml (range: 1.2×10^5 to 2.7×10^6 IU/ml).

With the TruGene HCV 5'NC Genotyping Kit, all of the Hungarian patients were found to be infected with genotype 1 (Table 8). The most common subtype was 1b (18 patients). Subtype 1a was present in only one patient, and in another one, genotype 1 could not be subtyped. The phylogenetic analysis verified the results of subtyping (Table 8). Subtype 1b was found in 19 patients with high sequence identity (Figure 1). In one patient, subtype 1c was detected.

Table 8. Distribution of HCV genotypes and subtypes in the studied populations

Genotype	Subtype	Number of patients	
		H - T[a]	H - P[b]
1		20	20
	1a	1	0
	1b	18	19
	1c	0	1
	Unidentified	1	0
2		0	0
3		0	0

[a] Number of Hungarian patients tested with the Trugene HCV 5'NC Genotyping assay
[b] Number of Hungarian patients tested with phylogenetic analysis

Of 72 borderline individuals with HCV reactivity, 65 (91.1%) were RT-PCR negative and 7 (8.9%) gave positive results. One patient became RT-PCR-positive after a delay. After initial RT-PCR positivity one other person gradually lost both HCV RNA and anti-HCV positivity. Three (6%) of 50 HCV antibody-negative sera proved to be HCV RNA-positive. One patient later seroconverted to HCV (Table 9).

Table 9. Comparison of HCV antibody test results and HCV RT-PCR results on the same serum samples

		HCV RT-PCR	
		Positive	Negative
HCV antibody	confirmed positive (1.5 S/CO**) (47 samples)	85% (40 samples)	15% (7 samples)
	borderline reactive (0.8-1.5 S/CO) (79 samples)	8.9% (7 samples)	91.1% (72 samples)
	negative (0.8 S/CO) (50 samples)	6%* (3 samples)	94% (47 samples)

* rate of RT-PCR-positive results in HCV antibody negative cases

** S/CO – sample rate/cutoff rate

Figure 1. Phylogenetic analysis of 5' NTR gene sequences of HCV genotypes / subtypes in 19 patients with chronic hepatitis C

4.2. EXAMINATION OF HEPATITIS G VIRUS

Different groups of blood donors and health-care workers without other hepatitis virus markers were tested for the presence of HGV antibodies. About a quarter of the control group without any other hepatitis virus markers were found to be seroconverted to the E2 antigen of HGV (26.3 %). The results summarized in Table 10 indicate that chronic HCV hepatitis and the HCV markers (both antibodies or RNA) also representing an increased risk of seroconversion to HGV. It should be mentioned that the chronic HCV-infected patients were enrolled into IFN therapy following the sample collection. IFN treatment too has been shown

to promote seroconversion to HGV virus. The low number of health-care workers with hepatitis virus markers tested prevented the calculation of P values.

Table 10. Anti-HGV in control and risk populations

Groups	**n**	**HBsAg**	**Anti-HCV**	**HCV PCR**	**Anti-HGV**
Control group	76	0	0	0	19 (26.3%)
Patients with chronic hepatitis C	48	0	48	48	27 (56%)
Anti-HCV-positive health-care workers	8	0	8	6	5 (62.5%)
HCV PCR positive health-care workers	6	0	6	6	3 (50%)
HBsAg positive health-care workers	10	10	0	0	3 (30%)

4.3. EXAMINATION OF ENTEROVIRUSES

When tenfold dilutions of two enterovirus strains, coxsackievirus B4 and echovirus 7, were tested by automated RNA extraction and real-time PCR on the LC instrument, the detection limit was found to be 0.1 $TCID_{50}$ (Fig. 2). With the dilution containing 0.01 $TCID_{50}$, the new molecular assay produced negative results.

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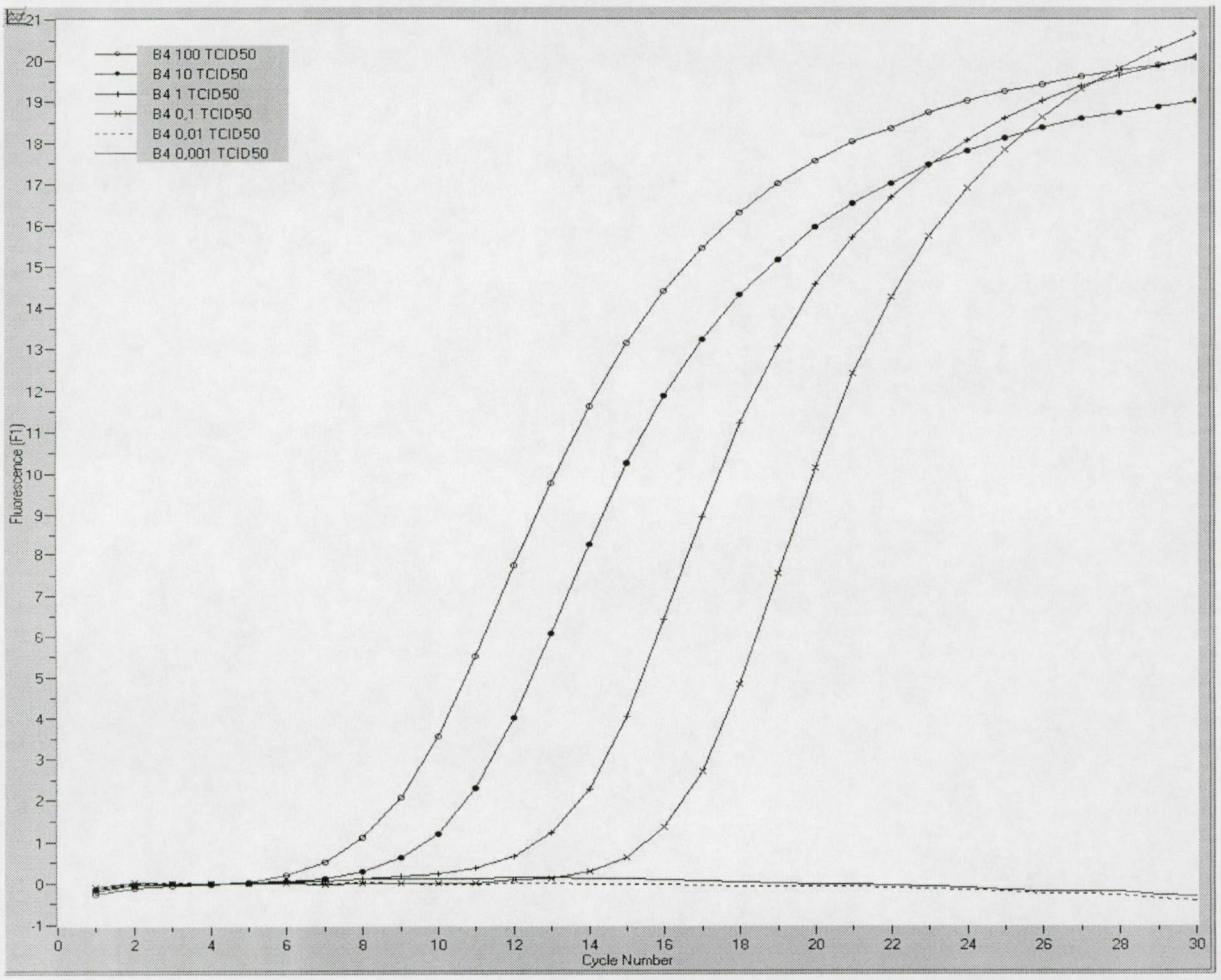
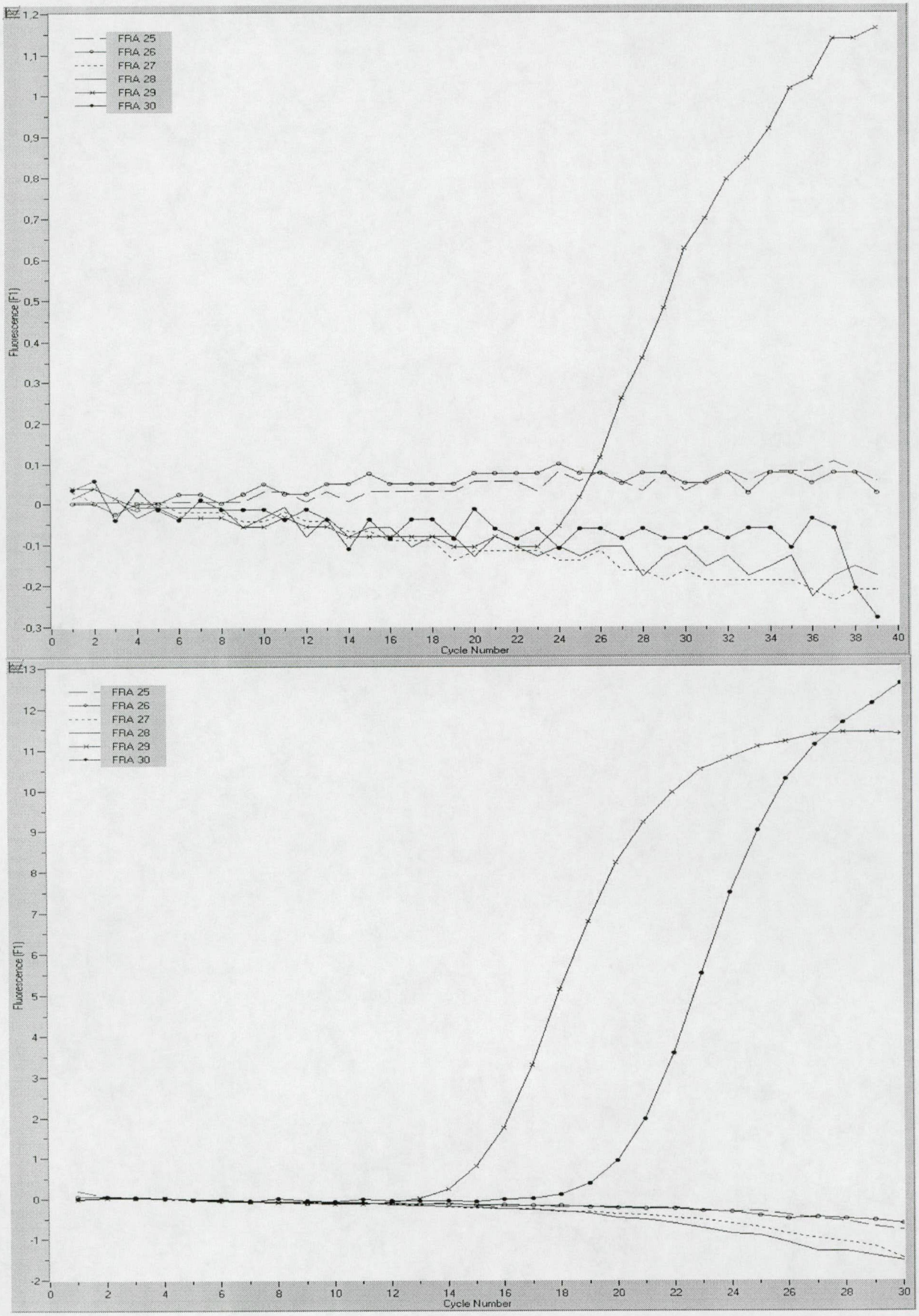
Figure. 2. LC PCR results on tenfold dilutions of coxsackievirus B4



When samples from the Third European Union Concerted Action Enterovirus Proficiency Panel were tested with automated RNA extraction and real-time PCR on the LC instrument and with the in-house assay, both molecular assays gave results identical to those expected on the basis of the results obtained by three reference laboratories using a total of four different molecular methods before distribution of the panel. For coxsackievirus A9, 1 $TCID_{50}$ (vial 3) and 0.1 $TCID_{50}$ (vial 2) could consistently be detected, whereas 0.01 $TCID_{50}$ (vial 1) was not detected at all. Only the samples containing coxsackievirus B5 (100 $TCID_{50}$; vial 7) and echovirus 6 (10 000 $TCID_{50}$; vial 5) were found positive. For echovirus 11, 10 000 $TCID_{50}$ (vial 9), 100 $TCID_{50}$ (vial 11), and 10 $TCID_{50}$ (vial 6) tested positive repeatedly. The only sample containing enterovirus 71 (10 $TCID_{50}$; vial 8) was consistently found positive.

From a total of 109 CSF specimens, 23 (21%) were repeatedly found to be positive by automated RNA extraction and real-time PCR on the LC instrument and by the in-house PCR assay and 82 proved to be negative by both molecular assays (Fig. 3).

Figure 3. LC PCR of clinical samples. A. Results after first-round PCR. B. Results after seminested PCR



Four samples yielded discrepant results: three of them were positive with the in-house assay and negative with the real-time assay, and the remaining one was positive with the real-time PCR assay and negative with the in-house PCR assay (Table 11). Upon repetition, all samples yielded the same results.

Table 11. Discrepant results

Patient no.	Age (yr)/sex[a]	In-house PCR assay (R)[b]	Real-time PCR (R)[b]	Enterovirus serology[c]	Final diagnosis
1	6/M	N	P (P)	ND	Aseptic meningitis
2	6/M	P (P)	N (N)	ND	Aseptic meningitis
3	7/M	P (P)	N (N)	IgG P; IgM N	Aseptic meningitis
4	30/M	P (P)	N (N)	ND	Aseptic meningitis

[a] F, female; M, male.

[b] R, repeat result; N, negative; P, positive.

[c] ND, not done; N, negative; P, positive; IgG, immunoglobulin G; IgM, immunoglobulin M.

The molecular assay based on automated RNA extraction and real-time PCR on the LC instrument proved to be quick and labor-saving. The whole procedure could be finished within 4 h. Automated RNA isolation with the MagNA Pure LC for the extraction of 32 samples could be completed within 105 min. This included a 15-min set-up of the MagNA Pure LC. The time required for the postelution protocol was 20 min. After centrifugation, the combined cycling and detection procedures took another 115 min, including 15 min for centrifugation and pipetting between the two rounds. No contamination was observed during the whole study. The in-house PCR assay took about 4 h. One hour was required for DNA extraction, 2 h for PCR, and another hour was required for detection by gel electrophoresis.

5. Discussion

5.1. HEPATITIS C VIRUS

The natural history of HCV infection is not well understood because of the difficulties of setting up and maintaining suitable studies. Much of the data on the natural history has come from retrospective studies of people with chronic liver disease and is biased towards populations who experience the more severe consequences of the disease. The results of studies of infectious diseases indicate that the prevalence, outcome and consequences of the disease all vary in different continents, in different countries, and even within the same country, and the reported data depend on sensitivity and specificityof the diagnostic methods applied.

5.1.1. Blood donors

The implementation of blood donor screening decreased the transmission of the virus via blood transfusion and the risk of post-transfusion hepatitis has declined from 1 in 5 000 to 1 in 200 000 [9].

In the present study, a relatively low prevalence of confirmed anti-HCV-positive blood donors was found in SouthHungary. It is important to bear in mind that adequate information on population HCV prevalenceis provided only data obtained from second or third-generation serology tests and confirmed by nucleic acid based technologies (NATs). Although prevalence of HCV infection in Hungary has been examined several times, the data are difficult to compare as different serologic assay confirmation methods and NATs were used in different populations [(90-93)]. Furthermore, the applied NATs also have to be validated, including sample collection and RNA extraction. The use of internal and external controls should guarantee the quality of the method and diminish or reduce the possibility of false-negative and false-positive results.

In our study, transmission of HCV was found to occur mainly by the nosocomial route. Other risk factors such as tattooing or intravenous drug use, however, must also be taken into consideration. The prevalence of anti-HCV antibodies among intravenous drug abusers in Hungary was recently shown to be 24% [(94)].

HCV RNA could be detected in only a small percentage (8.9%) of serum samples of borderline-positive cases in comparison with confirmed HCV antibody-positives (85%). According to the published data, low-level reactivity (0.8-10 S/CO) is connected false-positive results [(95)]. The diagnosis of HCV infection should never be based on a positive result

in a screening assay alone. HCV diagnosis is hampered by the restriction of antigens used in commercially available tests to genotype 1, which is predominantly found in the United States; these antigens do not necessarily represent viral genotypes found in other parts of the world [96]. Patients with indeterminate results and immunesupressed patients should always be tested by PCR. Thus, PCR is not only necessary to estimate the infectivity of an infected patient, but also often serves as a confirmatory assay. However, low-level viremia is found in chronically infected patients; a negative PCR result in a serum sample does not exclude ongoing HCV infection, nor does it prove spontaneous recovery from HCV [97].

5.1.2. Health-care workers

In our study, health-care workers do not seem to be at greater risk of acquiring HCV infection than in other studies [98, 99]. In the literature, more than 20 different pathogens are described as transmissible by syringes and injuries, though HCV is one of the greatest problems, as the infected individual has no signs of infection for a long time and in many cases (40%) the transmission of the HCV is not known. In our study, the prevalence of HCV infection was examined; HIV and HBV detection was performed in parallel with HCV screening but is not discussed here. The screening is continuing; the anti-HCV-positives are sent to the Hepatology Care Unit. Those infected with HCV, who perform invasive procedures are transferred to noninvasive treatment areas of the University. Further the safety provisions should be improved in order to prevent the nosocomial spreading of the virus as no vaccine is available against HCV.

5.1.3. Pregnant women and neonates

Until 1992, the main route of acquisition of HCV was post-transfusion or the application of unscreened hematological preparations. Since then, perinatal infection has become the dominant route among children. At the beginning of this study in 1996, mother-to-infant transmission seemed to be an important route of transmission of the virus [100]. Although the most important route of HCV infection is perinatal transmission among children nowadays, the prevalence is very low among those children who have no risk factors of possible HCV infection. There is evidence that the rate of spontaneous viral clearance is high (75%), though the follow-up of children at risk of perinatal HCV infection is important [101, 102]. Screening with the most effective methodologies amongst risk populations may play an important role in reducing the number of primary infections in the Hungarian population.

In the nonselected pregnant population, HCV infection is more frequent than among blood donors. The prevalence in our pregnant population corresponded to the West European and Japanese data, though screening of pregnants for HCV infection is not recommended, since

we have no effective method of preventing the perinatal transmission of the virus at present; however newly published data suggest that elective cesarean section can prevent HCV perinatal transmisson [12, 40]. In further studies on this topic, if the method proves to be reproducible, screening of pregnant women should be taken into consideration.

According to the recommendations EASL International Hepatitis C Consensus Conference, pregnancy is not contraindicated in women with HCV and perinatal transmission is restricted to infants whose mothers are viremic, but the association between the risk of neonatal infection, the maternal specific genotypes and the mode of delivery remains unclear. Breast-feeding is not contraindicated, since infection through breast-milk has not been observed in none HIV co-infected mothers, though breast-milk may contain viral particles [103].

Multicenter prospective studies are required to determine the natural history of HCV infection in perinatally infected children [104].

5.1.4. Children

Because of the low prevalence of anti-HCV-positive blood donors in SouthHungary, only a very small number of children who had received blood transfusions prior to implementation of anti-HCV blood donor screening were found to be infected with HCV.

After 2-10 years, persistent HCV infection was detected more frequently in children who had undergone neonatal transfusion than in the blood donor group. None of the children had signs of chronic HCV infection, but in two patients elevated serum ALT, AST and γGT levels were observed. Liver biopsy was not performed in these cases. The follow-up of these patients in hepatology care units is important because of the selection of patients for antiviral therapy and the risk of cirrhosis and HCC.

Chronic hepatitis seems to be more benign in the first two decades of life than in adults, but in cases involving of large inocula or infection with different genotypes, severe hepatic lesions may occur [17, 105]. Antiviral treatment in severe cases should be taken into account, but it is not recommended because of the lack of sufficient experience. As IFN treatment alone does not seem worthwhile in children as in adults, combination therapy with IFN and RB might be more effective when treatment is indicated, even with PEG-IFN [4].

According to recommendations on the pediatric population, it is not justified to exclude children from schools or social activities, though it is recommended that open wounds should be covered. In addition, these patients should be vaccinated against HAV and HBV, and should avoid contact with hepatotoxic drugs and alcohol. Adolescents should be provided with information concerning the possible risk of sexual transmission [104].

5.1.5. Patients with chronic hepatitis C

The natural history of HCV infection depends upon the route of transmission, the acquired virus inocula, the genotype, co-infection with HBV or HIV and host factors, such as immunestatus, age and gender [1]. In newly published studies, the prognosis seem to be more benign; one-third of the cases progress into more severe stages, and in 7-10% of these patients cirrhosis appears. The constant development of new and more effective antiviral drugs such as RB and PEG IFN offer recovery in an increasing number of cases.

HCV genotyping is useful for the solution of epidemiological questions and the development of vaccines against HCV. Furthermore, it has been shown to be beneficial in facilitating therapeutic decisions and strategies [106, 107].

In this study, the recently marketed TruGene HCV 5'NC Genotyping assay was employed has been found to be useful for the routine diagnostic laboratory [50, 105, 109]. Both the results obtained with the GeneObjects software and phylogenetic analysis revealed that all the Hungarian patients were infected with HCV genotype 1. The collected data correspond to those from previous studies, in which genotypes or serotypes were determined [27, 35, 110, 111]. In one of those earlier studies, 95% of all patients with genotype 1 were infected with subtype 1b [111]. The majority of those patients had received transfusions of blood and/or blood products. Phylogenetic analysis of genotype 1 sequences derived from the Hungarian patients revealed a high sequence identity among the isolates.

In comparison, 20 Austrian patients, 15 were found infected with genotype 1 with both methods. The remaining patients were infected with either genotype 3 or genotype 2. In a recent Austrian study, almost 75% of the patients were infected with HCV genotype 1, followed by genotypes 3 (16%), 4 (5%), and 2 (3%) [133]. The majority of patients with genotype 3 were drug abusers and all of the patients infected with genotype 4 originated from Middle East countries.

Before our work, HCV genotype analysis was carried out by RFLP, serotyping and probe specific hybridization in Hungary [27, 35, 110, 111]. Genotype detection with the Trugene HCV 5'NC Genotyping assay was found to be suitable in a routine diagnostic laboratory. It is mainly automated and can easily be performed by trained medical technicians. Comparison with other genotyping methods revealed that the Trugene HCV 5' Genotyping assay is practical for routine laboratory use and can detect HCV genotypes safely [50, 108, 109]. Classification of subtypes, however, was not possible in all HCV isolates. This might be due to the short amplification product within the highly conserved genome region (only one nucleotide difference at position 99 for the discrimination of subtypes 1a and 1b). In those

cases, phylogenetic analysis may help with the determination of the HCV subtype. The discrimination between subtypes 1a and 1b is not important for therapeutic decisions because the therapeutic strategies differ only between patients infected with subtype 1 and patients infected with non-1 subtypes [112].

5.2. EXAMINATION OF HEPATITIS G VIRUS

In Hungary, 26.3% of the healthy control population have already been seroconverted from HGV/infected. This correlates with other published Hungarian data. In Hungary, Takács et al. investigated the HGV antibody prevalence among healthy persons. In their control group, the rate of HGV antibody positivity was similar to that for our conrol group. The prevalence seems to be high relative to the international data [111]. Among patients with chronic hepatitis C and chronic hepatitis B, HGV antibody could be found at higher rates (56%, 50% and 30%). Transfusion or surgery before the implementation of blood donor screening could be found in the case histories. HGV infection seems to occur at higher rates amongst HCV and HBV-infected patients, but definite evidence linking it to a specific disease or illness is lacking [113].

5.3. EXAMINATION OF ENTEROVIRUSES

In the present study, a new molecular assay based on automated DNA extraction with the MagNA Pure LC and real-time PCR on the LC instrument was evaluated and compared with a conventional in-house PCR assay. The detection limit of the new molecular assay was found to be similar to that of the in-house assay indicating that the two both extraction methods may result in similar amounts of enterovirus RNA.

In Hungary, the last of the three countrywide epidemics was caused by a "prime " strain of echovirus 11 (echovirus 11') in 1989 [69, 114-117]. Mass vaccination with monovalent OPV type 1 stopped the epidemic within one week [86]. Enterovirus infection can be health-threatening among pregnant women and newborns, although there is no confirmed association with congenital abnormalities. In young infants, enteroviral meningitis is often confused with illnesses caused by bacteria and herpesvirus, resulting in thousands of children being hospitalized and incorrectly treated with antibiotics and anti-herpes drugs.

In this study, discrepant results may have emerged from amplification failures of enterovirus serotypes, contamination problems, and nonspecific amplification products. It must be taken into account, however, that enteroviruses are "quasispecies". The international panels were prepared in permanent human or monkey tissue culture cell lines, which are able

to "homogenize" the quasispecies nature of the viruses. It has recently been proven that even the most conservative (5'NTR) regions of enteroviruses might carry significant sequence differences [118, 119]. In the clinical diagnostics of CSF samples, the "quasispecies nature" of the virus may prevent amplification due to point mutations. A direct cloning procedure has therefore been developed to clone all sequence variants present in a diagnostic sample [119].

The primer and probe sets most frequently used for the detection of enteroviruses in clinical specimens are those reported by Chapman et al. and Rotbart et al. [88, 120, 121]. For the new molecular assay, primers with minor modifications of those first described by Rotbart were employed. Primers and probes identical to or with minor modifications to those first described by Rotbart were found to be truly panreactive with human enteroviruses [79, 122, 123]. These primers only failed to amplify echoviruses 22 and 23, which have been reported to be genetically distinct picornaviruses and are rarely isolated from CSF [124-127]. Specific PCR products were not detected when a variety of non-enterovirus meningitis pathogens were tested; however, amplification products were detected from RNA extracts of five rhinovirus serotypes [79]. Cross-reactivity between rhinoviruses and enteroviruses is produced by the sequence homology between these two picornavirus groups. This cross-reactivity, however, is of minor importance in the testing of CSF specimens, as there are no published reports of the isolation of rhinovirus from CSF specimens. This would have an important impact on the testing of nasal or pharyngeal swabs. For the in-house PCR assay, primers with minor modifications of those first described by Chapman et al. were employed. These primers have also been shown to be panreactive with human enteroviruses and to fail to amplify echoviruses 22 and 23 [88].

It has been demonstrated that the probability of false-positive results because of contamination increases in relation to the number of hands-on manipulations involved in the sample processing [31, 128]. The new molecular assay includes RNA extraction with the MagNA Pure LC, a fully automated nucleic acid extraction instrument. This instrument has recently been shown to be reliable and did not produce contamination during a study on the automated extraction of herpes simplex DNA [129]. The remaining weakness in the design of the new molecular assay, which could lead to false-positives, may be the pipetting steps between the first-round PCR and the second-round PCR. In the future, this step may be avoided by a two-round PCR on the LC, using a single closed capillary to exclude the possibility of amplification product carry-over [130].

The detection formats of the LC technology include the general detection of double stranded DNA (SYBR® Green technology), which corresponds to the gel electrophoresis of

in-house assays, and the specific detection of the target sequence by using a TaqMan probe or hybridization probes [131]. Probes prevent false-positive results due to nonspecific amplification products and to guarantee specificity of the results. Therefore, probes should always be employed for molecular assays in the routine diagnostic laboratory.

The required time for testing 32 samples was similar with the two molecular assays. If less than 8 specimens are extracted, the extraction kit used for the in-house molecular assay will be the quicker method. However, it must be taken into consideration that the hands-on time for extraction is only 15 min when the MagNA Pure LC is used. As compared with the in-house assay, a 50% increase in cost was observed with the LC assay.

In summary, the new molecular assay based on extraction with MagNA Pure LC and real-time PCR on the LC instrument proved suitable for the routine diagnostic laboratory. As compared to a conventional in-house assay, it was found to be less labor-intensive and easy to use. Because of the lower number of manipulations, there may be a lower probability of false-positive results due to contamination.

6. Concluding Remarks

6.1. EXAMINATION OF HCV INFECTION

The prevalence of chronic HCV is low in Hungary and transmission by blood transfusion can almost be ruled out in South Hungary nowadays. The implementation of NAT or the newly developed antigen detecting system would reduce the risk of HCV transmission through blood or blood products even further. Because of the low prevalence of anti-HCV-positive blood donors, pregnant women and health-care workers in South Hungary, only a very small number of children were infected with HCV; they had received blood transfusion prior to the introduction of anti-HCV blood donor screening. The diagnosis of HCV infection should never be based on a positive result in a screening assay alone. The results of this study reflect the present situation regarding the prevalence of HCV genotypes in South Hungary, with genotype 1 the predominant subtype. Comparison with genotyping results in South-east Austria, where 15 were found to be infected with genotype 1 and the remaining 5 patients were infected with either genotype 3 or genotype 2, reflect the present situation regarding the prevalence of HCV genotypes in South Hungary and South-east Austria, with genotype 1 the predominant subtype in both regions. This genotype has the worst prognosis and an adverse response to antiviral treatment. The largely automated sequencing assay for the determination of HCV genotypes proved useful for a high-throughput routine diagnostic laboratory.

6.2. EXAMINATION OF HGV INFECTION

The anti-E2 prevalence in high-risk groups, such as chronic HCV-infected patients and health-care workers, seems to be high, though definitive evidence linking it to a specific disease or illness is lacking.

6.3. EXAMINATION OF ENTEROVIRUSES

A qualitative molecular assay based on automated RNA extraction on the MagNA Pure LC instument and real-time PCR with LC system was evaluated. The new molecular assay allows rapid detection of enteroviruses in CSF. It was found to be labor-saving and displayed sufficient sensitivity.

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8. References

1. Specter S. Viral hepatitis: diagnosis, therapy and prevention. Humana Press, Totowa, New Jersey 1999; 2-3.
2. Tanaka Y., Primi D., Wang R.Y., Umemura T., Yeo A.E., Mizokami M., Alter H.J., Shih J.W. Genomic and molecular evolutionary analysis of a newly identified infectious agent (SEN virus) and its relationship to the TT virus family. J Infect Dis 2001; 183: 359-362.
3. Bowden D.D., Moaven L.D., Locarini S.A. New hepatitis viruses: are there enough letters in the alphabet? Med J Australia 1996; 164: 87-89.
4. Recommendation for prevention and control of hepatitis C virus (HCV) HCV-related chronic disease Morb Mortal Wkly Rep 1998:47 (RR-19).
5. Seeff L.B., Hollinger F.B, Alter H.J, Wright E.C., Cain C.M.B., Buskell Z.J., Ishak K.G., Iber F.L., Toro D., Samanta A., Koretz R.L., Perrillo R.P., Goodman Z.D., Knodell R.G., Gitnick G., Morgan T.R., Schiff E.R., Lasky S., Stevens C., Vlahcevic R.Z., Weinshel E., Tanwandee T., Lin H.J., Barbosa L. Long-term mortality and morbidity of transfusion-associated non-A, non-B and type C hepatitis: a National Heart, Lung, and Blood Institute Collaborative Study. Hepatology 2001; 33: 455-463.
6. Lohmann V., Korner F., Koch J., Herian U., Theilman L., Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999; 285: 110-113.
7. Kurtz J.B., Boxall E., Qusir N., Shirley J., Coleman D., Chandler C. The diagnostic significance of an assay for 'total' hepatitis C core antigen. J Virol Methods 200; 96: 127-32.
8. Lee S.R., Wood C.L., Lane M.J., Francis B., Gust C., Higgs C.M., Nelles M.J., Polito A., DiNello R., Achord D. Increased detection of hepatitis C virus infection in commercial plasma donors by a third-generation screening assay. Transfusion 1995; 35: 845-849.
9. Pawlotsky JM. Diagnostic tests for hepatitis C. J Hepatol 1999; 31: 71-79.
10. Pillonel J., Saura C., Courouce A.M. Screening of viral markers for HIV, HBV and HCV infections in blood donors in France and residual risk of viral transmission by blood transfusion. Eurosurveillance 1998; 3: 76-79.
11. Bortolotti F, Resti M, Giacchino R, Azzari C, Gusetti N., Crivellaro C., Zancan L, Azzari C, Gussetti N, Tasso L, Faggion S. Hepatitis C virus infection and related liver disease in children of mothers with antibodies to the virus. J Pediatr 1997; 130: 990-3.
12. Tajiri H., Miyoshi Y., Funada S., Etani Y., Abe J., Onodera T., Goto M., Funato M., Ida S., Noda C., Nakayama M., Okada S. Prospective study of mother-to-infant transmission of hepatitis C. Pediatr Infect Dis J 2001; 20: 10-14.
13. Committe on Infectious Diseases, American Academy of Pediatrics. Hepatitis C virus infection. Pediatrics 1998; 101: 481-5.
14. Zanetti A.R., Tanzi E., Newell M.L. Mother-to-infant transmission of hepatitis C virus. J Hepatol 1999; 31: 96-100.
15. Sokal E.M., Bortolotti F. Update on prevention and treatment of viral hepatitis in children. Curr Opin Pediatrics 1999; 11: 384-389.

16. Thomas S.L., Newell M.L., Peckham C.S., Ades A.E., Hall A.J. A review of hepatitis C virus (HCV) perinatal transmission: risks of transmission to infants born to mothers with and without HCV viraemia or human immunodeficiency virus infection. Int J Epidemiol 1998; 27: 108-117.
17. Vogt M., Lang T., Frösner G., Klinler C., Sendl A.F., Zeller A., Wiebecke B., Langer B., Meisner H., Hess J. Prevalence and clinical outcome of hepatitis C infection in children who underwent cardiac surgery before the implementation of blood-donor screening. N Engl J Med 1999; 341: 866-70.
18. Schwimmer J.B., Balistereri W.F. Transmission, natural history, and treatment of hepatitis C virus infection in the pediatric population. Sem Liv Dis 2000; 20: 37-46.
19. Grasselly M., Csákváry V., Schneider F. Újszülöttkorban vércserében és többszörös transzfúzióban részesült gyermeke hepatitis C vírus seroconversiójának gyakorisága. Gyermekgyógyászat 1997; 6: 666-669. (in Hungarian)
20. Strickland D.K., Riely C.A., Patrick C.C., Jones-Wallace D., Boyett J.M., Waters B., Fleckenstein J.F., Dean P.J., Davila R., Caver T.E., Hudson M.M. Hepatitis C infection among survivors of childhood cancer. Blood 2000; 95: 3065-70.
21. Davison S.M., Skidmore S.J., Collingham K.E., Irving W.L., Hubscher S.G., Kelly D.A. Chronic hepatitis in children after liver transplantation: role of hepatitis C virus and hepatitis G virus infections. J Hepatol 1998; 28: 764-70.
22. Pereira B.J., Levey A.S. Hepatitis C virus infection in dialysis and renal transplantation. Kidney Int 1997; 51: 981-99.
23. Liang T.J., Rehermann B., Seeff L.B., Hoofnagle J.H. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 2000; 132: 296-305.
24. Tong M.J., El-Farra N.S., Reikes A.R. Co R.L. Clinical outcomes after transfusion-associated hepatitis C. N Engl J Med 1995; 332: 1463-1466.
25. Pár A. Chronic hepatitis C: virologic and immunologic aspects and questions therapy. Orv Hetil 1997; 138: 1465-76.
26. Pár A. Diagnosis and management of chronic hepatitis C. Can J Gastroenterol 2000; 14: 83B-88B.
27. Pár A., Gervain J., Gógl Á. Hepatitis C virus infection: pathogenesis, diagnosis and treatment. Scand J Gastroenterol Suppl 1998; 228, 107-14.
28. Di Bisceglie A.M., McHutchison J., Rice C.M. New therapeutic strategies for hepatitis C. Hepatology 2002; 35: 224-231.
29. Simmonds P., Mutimer D., Follett E.A.C. Hepatitis C. In Collier L. Topley, Wilson's Microbiology and Microbial Infections, Ninth Edition, Arnold, UK 1999; 36.
30. Damen M., Cuypers H.T., Zaaijer H.L., Reesink H.W., Schaasberg W.P., Gerlich W.H. International collaborative study on the second EUROHEP HCV-RNA reference panel. J Virol Methods 1996: 58: 175-185.
31. Kwok S, Higuchi R. Avoiding false positives with PCR. Nature 1989; 339, 237-238.
32. Valentine-Thon E. Borderline and/or discordant Cobas Amplicor HCV test version 2.0 results: clinical significance. J Clin Virol 2001; 20: 77-80.

33. Ross R.S., Viazov S.O., Hoffmann S., Roggendorf M. Performance characterisctics of a transcription-mediated nucleic acid amplification assay for qualitative detection of hepatitis C virus RNA. J Clin Lab Anal 2001; 15: 308-13.
34. Müller Z., Deák J., Horányi M., Szekeres É., Nagy I., Ozsvár Z., Nagy E., Lonovics J., Gál G. The detection of hepatitis C virus in South Hungary. Journal of Clinical Virology 2001; 20: 81-83.
35. Gervain J., Simon G Jr., Papp I., Szabóné BK. Determination of the type and subtype of the hepatitis C virus in chronic viral hepatitis patients in Hungary Orv Hetil. 2001; 142: 1315-9. (in Hungarian)
36. Sárváry E., Varga M., Nemes B., Kobori L., Zalka A., Sulyok B., Görög D., Fehérvári I., Járay J., Halmos O., Alföldy F., Tóth A., Lakatos M., Perner F. Qualitative and quantitative detection of hepatitis C virus RNA by PCR technique. Monitoring of viral copies after liver transplantation Orv Hetil. 2001; 142: 939-42. (in Hungarian)
37. Van der Poel C.l. Hepatitis C virus and blood transfusion: past and present risks. J Hepatol 1999; 31: 101-6.
38. Sun R., Schilling W., Jayakar H., Ku J., Wang J., Rosenstraus M., Spadoro J. Simultaneous extraction of hepatitis C virus (HCV), hepatitis B virus, and HIV-1 from plasma and detection of HCV RNA by a reverse transcriptase-polymerase chain reaction assay designed for screening pooled units of donated blood. Transfusion 1999; 39: 1111-1119.
39. Petrik J. Microarray technology: the future of blood testing? Vox Sang 2001; 80: 1-11.
40. Gibb D.M., Goodall R.L., Dunn D.T., Healy M., Neave P., Cafferkey M., Butler K. Mother-to-child transmission of hepatitis C virus: evidence for preventable peripartum transmission. Science 2000; 356: 904-907.
41. Podzorski R.P. Molecular testing in the diagnosis and management of hepatitis C virus infection. Arch Pathol Lab Med 2002; 126: 285-290.
42. Choo Q-L, Richman K.H., Han J.H., Berger K., Lee C., Dong C., Gallegos C., Coit D., Medina-Selby A., Barr P.J., Weiner A.J., Bradley D.W., Kou G., Houghton M. Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci USA 1991; 88: 2451-5.
43. Okamoto H., Kurai K., Okada S., Yamamoto K., Lizuka H., Tanaka T., Fukuda S., Tsuda F., Mishiro S. Full-length of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology 1992; 188: 331-341.
44. Simmonds P., Alberti A., Alter F., Bonino F., Bradley D.W., Brechot C., Brouwer J.T., Chan S.W., Chayama K., Chen D.S. A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology 1994; 19: 1321-1324.
45. Simmonds P. Viral heterogeneity of the hepatitis C virus. J Hepatol 1999; 31: 54-60.
46. Simmonds P., Holmes E.C., Cha T.A., Chan S.W., McOmish F., Irvine B., Beall E., Yap P.L., Kolberg J., Urdea M.S. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol 1993; 74: 2391-2399.
47. Widell A., Shev S., Mansson S., Zhang Y.Y., Foberg U., Norkrans G., Fryden A., Weiland O., Kirkus J., Nordenfelt E. Genotyping of hepatitis C virus isolates by a modified polymerase chain reaction assay using type specific primers: epidemiological application. J Med Virol 1994; 44: 272-279.

48. Davidson F., Simmonds P., Ferguson J.C., Jarvis L.M., Dow B.C., Follet E.A., Seed C.R., Krusius T., Lin C., Medgyesi G.A. Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequencing amplified from the 5` non-coding region. J Gen Virol 1995; 76: 1197-1204.
49. Stuyver L., Wyseur A., Van Arnhem W., Hernandez F., Maertens G. Second-generation line probe assay for hepatitis C virus genotyping. J Clin Microbiol 1996; 34: 2259-2266.
50. Ross R.S., Viazov S.O., Holtzer C.D., Beyou A., Monnet A., Mazure C., Roggendorf M. Genotyping of hepatitis C virus isolates using CLIP sequencing. J Clin Microbiol 2000; 38: 3581-3584.
51. Mondelli M.U., Silini E. Clinical significance of hepatitis C virus genotypes. J Hepatol 1999; 31: 65-70.
52. Power J.P., Lawlor E., Davidson F., Holmes E.C., Yap P.L, Simmonds P. Molecular epidemiology of an outbreak of infection with hepatitis C virus in recipients of anti-D immunoglobulin. Lancet 1995; 345: 1211-1213.
53. Civeira M-P., Prieto J. Early predictors of response to treatment in patients with chronic hepatitis C. J Hepatol 1999; 237-243.
54. Simons J.N., Leary T.P., Dawson G.J., Pilot-Matias T.J., Muerhoff A.S., Schlauder G.G., Desai S.S.M., Mushawar I.K. Isolation of novel virus-like sequences associated with human hepatitis. Nat Med 1995; 1: 564-570.
55. Linnen J., Wages J. Jr, Zhang-Keck Z.Y., Fry K.E., Krawaczynski K.Z. Molecular cloning and disease association of hepatitis G virus: A transfusion transmissible agent. Sience 1996; 271: 505-510.
56. Smith D.B., Cuceanu N., Davidson F. Discrimination of hepatitis G virus/GBV-C geographical variants by analysis of the 5 non-coding region. J Gen Virol 1997; 78: 1533-1537.
57. Wejstal R., Manson A.S., Widell A., Norkrans G. Perinatal transmission of hepatitis G virus (GB virus type C) and hepatitis C virus infections- a comparison. Clin Infect Dis 1999; 28: 816-821.
58. Yeo A.E.T., Matsumoto A., Shih J.W., Alter H.J. Goedert J.J. Prevalence of hepatitis G virus in patients with hemophilia and their steady female sexual partners. Sex Trans Dis 2000; 27: 178-182.
59. Lefrere J.J., Lerable J., Mariotti M., Bogard M., Thibault V. Lessons from a multicentre study of the detectability of viral genomes based on a two-round quality control of GB virus C (GBV-C)/ hepatitis G virus (HGV) polymerase chain reaction assay. J Vir Meth 2000; 85: 117-123.
60. Dille B.J., Surowy TK, Gutierrez R.A., Coleman P.F., Knigge M.F. An ELISA for detection of antibodies to the E2 protein of GB virus C. J Infect Dis 1996; 225: 293-300.
61. Kiyosawa K., Tanaka E. GB virus C/hepatitis G virus. Intervirology 1999; 42: 185-195.
62. Takács M., Rusvai E., Brojnás J., Tóth G., N. Szomor K., Tóth E., Szendrői A., Mezey I., Berencsi Gy. A hepatitis G- és TT-vírus diagnosztikájának bevezetése Magyarországon. Lege Artis Medicinae. 2001; 11: 274-282.
63. Müller Z., Deák J., Nagy E. Hepatitis G vírus – pathogén vagy endosymbionta? Klinikai Mikrobiológia és Infektológia (in press) (in Hungarian)
64. Pérez-Grazia T., Galán F., Girón-Gonzalez J.A., Lozano A., Benavides B., Fernandez E., Rodrigues-Iglesias M. Detection of hepatitis G virus (HGV) RNA and antibodies to the HGV envelope protein E2 in a cohort of hemodialysis patients. J Clin Microbiol 2000; 38: 4277-4279.
65. Alter H.J., Nakatsuji Y., Melpolder J., Wages J., Wesley R. The incidence of transfusion-associated hepatitis G virus infection and its relation to liver disease. N Engl J Med 1997; 336: 747-754.

66. Mushawar I.K. Recently discovered blood-borne viruses: are they hepatitis viruses or merely endosymbionts? J Med Virol 2000; 62: 399-404.

67. King AMQ, Brown F, Christian P, Hovi T, Hyypiä T, Knowles NJ, Lemon SM, Minor PD, Palmenberg AC, Skern T, Stanway G. Picornaviridae. In: Van Regenmortel MHV, Fauquet CM, Bishop DHL, Calisher CH, Carsten EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB (eds) Virus Taxonomy Seventh Report of the International Commitee for the Taxonomy of Viruses. Academic Press, New York, San Diego, 2000; 657-673.

68. Welch, J.B., McGowan K., Searle B., Gillon J., Jarvis L.M., Simmonds P. Detection of enterovirus viraemia in blood donors. Vox Sang 2001; 80: 211-215.

69. Berencsi Gy. Az enterovírusok molekuláris biológiai tulajdonságai és a hazai járványok jellegzetességei. 2001; (in Hungarian)

70. Minor P.D. Morgan.Capter P., Muir P. Enteroviruses. In: Zuckerman A.J., Banatvala J.E., Pattison J.R. (eds) Principles and Practice of Clinical Virology. 4ed Wiley, 2000; 14: 427-449.

71. Pulli T., Koskimies P., Hyypiä T. Molecular comparison of coxsackie A virus serotypes. Virology 1995; 212: 30-38.

72. Poyry T., Kinnunen L., Hyypiä T., Brown B., Horsnell C., Hovi T., Stanway G. Genetic and phylogenetic clustering of enteroviruses. J Gen Virol 1996; 77: 1699-717.

73. Hyypiä T., Stanway G. Classification of enteroviruses based on molecular and biological properties. J Gen Virol 1997; 78: 1-11.

74. Chonmaitree T., Menegus M.A., Powell K.R. The clinical relevance of CSF viral culture. A two-year experience with aseptic meningitis in Rochester, New York. JAMA 1982; 247: 1843-1847.

75. Rotbart H.A. Nucleic acid detection systems for enteroviruses. Clin Microbiol Reviews 1991; 4: 156-168.

76. Rotbart H.A., Romero J.R. Laboratory diagnosis of enteroviral infections. In: Rotbart H, editor. Human enterovirus infections. American Society for Microbiology, Washington, DC, 1995; 401-410.

77. Andreoletti L., Blassel-Damman N., Dewilde A., Vallee L., Cremer R., Hober D., Wattre P. Comparison of use of cerebrospinal fluid, serum, and throat swab specimens in diagnosis of enteroviral acute neurological infection by a rapid RNA detection PCR assay. J Clin Microbiol 1998; 36: 589-591.

78. Gorgievski-Hrisoho M., Schumacher J.D., Vilimonovic N., Germann D., Matter L. Detection of enteroviruses by RT-PCR in cerebrospinal fluid during a summer outbreak of aseptic meningitis in Switzerland. J Clin Microbiol 1998; 36: 2408-2412.

79. Kessler H.H., Santner B., Rabenau H.F., Berger A., Vince A., Lewinski C., Weber B., Pierer K., Stuenzner D., Marth E., Doerr H.W. Rapid diagnosis of enterovirus infection by a new one-step reverse transcription-PCR assay. J Clin Microbiol 1997; 35: 976-977.

80. Pozo F., Casas I., Tenorio A., Trallero G., Echevarria J.E. Evaluation of a commercially available reverse transcription-PCR assay for diagnosis of enteroviral infection in archival and prospectively collected cerebrospinal fluid specimens. J Clin Microbiol 1998; 36: 1741-1745.

81. Searle K., Dirmeier D., Metzger C., Enders G. A semi-automated PCR-ELISA as an alternative to virus isolation in cell culture for the routine diagnosis of EV infection. Clin Lab 1997; 43: 659-664.

82. Carroll K.C., Taggart B., Robinson J., Byington C., Hillyard D. Evaluation of the Roche AMPLICOR enterovirus PCR assay in the diagnosis of enteroviral central nervous system infections. J Clin Virol 2000; 19: 149-156.

83. Kessler H.H., Mühlbauer G., Stelzl E., Daghofer E., Santner B.I., Marth E. Fully automated Nucleic acid extraction: MagNA Pure LC. Clinical Chemistry 2001; 47: 1124-1126.

84. Wittwer C.T., Ririe K.M., Andrew R.V., David D.A., Gundry R.A., Balis U.J. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques 1997; 22: 176-181.

85. Dömök I. Enterovirus infections: Poliomyelitis. In: Robinson D (ed). Epidemiology and the community control of disease in warm climate counties. 2nd ed., Churcill-Livingstone-Edinburgh-London-Melbourne-New York 1985.

86. El-Sageyer M.M., Szendői A., Hütter E., Új M., Szűcs G., Mezey I., Tóth I., Kátai A., Kapiller Z., Páll G., Petrás G., Szalay E., Mihály I., Gourova S., Berencsi G. Characterisation of an echovirus type 11' (prime) epidemic strain causing haemorrhagic syndrome in newborn babies in Hungary. Acta Virol 1998; 42: 157-166.

87. Sawyer M.H. Enterovirus infections: diagnosis and treatment. Curr Opin Pediatr 2001; 13: 65-69.

88. Chapman N.M., Tracy S., Gauntt C.J., Fortmueller U. Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic acid hybridization. J Clin Microbiol 1990; 28: 843-850.

89. Severini G.M., Mestroni L., Falaschi A., Camerini F., Giacca G. Nested polymerase chain reaction for high-sensitivity detection of enteroviral RNA in biological samples. J Clin Microbiol 1993; 31: 1345-1349.

90. Barna T.K., Ozsvár Zs., Szendrenyi V., Gál G. Hepatitis C virus antibody in the serum of blood donors. Orv Hetil 1996; 137: 507-11. (in Hungarian)

91. Illés M., Szontágh L., Gál Gy. Hepatitis-B és –C markerek előfordulása hematológiai, dializált urémiás betegek és véradók szérumában. Tanszfúzió 1992; 25: 11-14. (in Hungarian)

92. Baynai B., Kraszits I. Hepatitis C pozitív debreceni véradók utóvizsgálatai. Transzfúzió 1999; 32: 127-131. (in Hungarian)

93. Gervain J., Gógl Á., Szabó G., Simon G Jr. How great is the risk of personal contact in the transmission of hepatitis virus infection? Orv Hetil 1997; 138: 607-9. (in Hungarian)

94. Osztrogonácz H., Gerevich J., Horváth G., Tolvaj Gy., Dávid K. Examination of the prevalence of chronic viral hepatitis infections in drug users. Orvosi Hetilap 2000, 141: 715-718. (in Hungarian)

95. Polywka S., Schröter M., Feucht H., Zöllner B., Laufs R. Relevance of reactivity in commercially avaible hepatitis C virus antibody assay. J Clin Microbiol 2001; 39: 1665-1668.

96. Dhaliwal S.K., Prescott L.E., Dow B.C., Davidson F., Brown H., Yap P.L., Follet E.A.C., Simmonds P. Influence of viraemia and genotype upon serological reactivity in screeening assays for antibody to hepatitis C virus. J Med Virol 1996; 48: 184-190.

97. Feucht H.H., Zöllner B., Schröter M., Polywka S., Buggisch P., Nolte H., Laufs R. High rate of chronicity in HCV infection determined by antibody confirmatory assays and PCR in 4110 patients during long-term follow-up. J Clin Virol 1999; 13: 43-51.

98. Mihály I., Telegdy L., Ibrányi E., Lukács A., Rókusz L., Bánkuti EE., Dóczy J. Prevalence, genotype distribution and outcome of hepatitis C infections among the employees of the Hungarian Central Hospital for Infectious Diseases. J Hosp Infect. 2001; 49: 239-244.

99. Lehel F., Csajbókné B.M., Hangyal Z. Study of viral infections among hospital personnel. Orv Hetil 1998; 139: 115-9.

100. Berencsi Gy., Takács M., Garamvölgyi E. A hepatitis C vírus genetikai és biológiai tulajdonságai. Transzfúzió 1993; 26: 49-57. (in Hungarian)

101. Ceci O., Margiotta M., Marello F., Francavialla R., Loizzi P., Francavilla A., Mautone A., Impedovo L., Ierardi E., Mastroianni M., Bettocchi S., Selvaggi L. Perinatal transmission of hepatitis C virus in a cohort of 2,447 HIV-seronegative pregnant women: a 24-month prospective study. J Pediatr Gastroenterol Nutr 2001; 33: 570-575.

102. Mihály I., Rédei E., Ibrányi E., Tímár L., Trethon A., Lukács A. Perinatal transmission of hepatitis C virus. Act Microbiol Immunol Hung 1999; 46: 426-427.

103. Zanetti A.R., Tanzi E., Paccagnini S., Principi N., Pizzocolo G., Caccamo M.L., D'Amico E., Cambie G., Vecchi L. Mother-to-infant transmission of hepatitis C virus. Lombardy Study Group on Perinatal HCV Transmission. Lancet 1995; 345: 289-91.

104. Committe on Infectious Diseases, American Academy of Pediatrics. Hepatitis C virus infection. Pediatrics 1998;101: 481-5.

105. Badizadegan K., Jonas M.M., Ott M.J., Nelson S.P., Perez-Atayde A.R. Histopathology of the liver in children with chronic hepatitis C viral infection. Hepatology 1998; 28: 1416-1423.

106. Poynard T., Bedossa P., Chevallier M., Mathurin P., Lemonnier C., Trepo C., Couzigou P., Payen J.L., Sajus M., Costa J.M. A comparison of three interferon alfa-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. Multicenter Study Group. N Engl J Med 1995; 332: 1457-62.

107. Davis G.L., Esteban-Mur R., Rustgi V., Hoefs J., Gordon S.C., Trepo C., Shiffman M.L., Zeuzem S., Craxi A., Ling M.H., Albrecht J. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. N Engl J Med 1998; 339: 1493-1499.

108. Ansaldi F., Torre F., Bruzzone B.M., Picciotto A., Crovari P., Icardi, G. Evaluation of a new hepatitis C virus sequencing assay as a routine method for genotyping. J Med Virol 2001; 63: 17-21.

109. Halfon P., Trimoulet P., Bouliere M., Khiri H., Lédinghen V., Couzigou P., Feryn J. M., Alcaraz P., Renou C., Fleury H.J.A., Ouzan, D. Hepatitis C virus genotyping based on 5' Noncoding Sequence Analysis (Trugene). J Clin Microbiol 2001; 39: 1771-1773.

110. McOmish F., Yap Pl. L., Dow B. C., Follett E.A.C., Seed C., Keller A. J., Cobain T.J., Krusius T., Kolho E., Naukkarinen R., Lin C., Lai C., Leong S., Medgyesi G. A., Héjjas M., Kiyokawa H., Fukada K., Cuypers T., Saeed A.A., Al-Rasheed A.M., Lin M., Simmonds, P. Geographic distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. J Clin Microbiol 1994; 32: 884-892.

111. Jarvis L.M., Ludlam C.A., Ellender J.A., Nemes L., Field S.P., Song E., Chuansumrit A., Preston F.E., Simmonds P. Investigation of the relative infectivity and pathogenicity of different hepatitis C virus genotypes in hemophiliacs. Blood 1996; 87: 3007-3011.

112. Mondelli M.U., Silini E. Clinical significance of hepatitis C virus genotypes. J Hepatol 1999; 31: 65-70.

113. Mphahlele M.J., Lau G.K.K., Carman W.F. HGV: The identification, biology and prevalence of an orphan virus. Liver 1998; 18: 143-155.

114. Dömök I., Molnár E. Meningiencephalitits cases among newborn babies in connection with the Bornholm epidemic in 1958. Orv Hetil 1960; 101: 593-597. (In Hungarian)

115. Dömök I. Report on A WHO collaborative study. Markers of poliovirus strains isolated from cases temporally associatedwith the use of live poliovirus vaccine. J Biol Stand 1981; 9: 163-184.

116. Nagy G., Takátsy S., Kukán E., Mihály I., Dömök I. Virological diagnosis of enterovirus type 71 infections: experiences gained during an epidemic of acute CNS diseases in Hungary in 1978. Arch Virol 1982; 71: 217-227.

117. Szirmai Z., György E., Polgár M., Benkovits S. Myocarditis in the newborn infant caused by coxsackie B2 enterovirus. Orv Hetil 1982; 123: 277-282. (in Hungarian)

118. Szendrői A., El-Sageyer M., Takács M., Mezey I., Berencsi Gy. Nucleotide sequences and mutations of the 5'-nontranslatedregion (5'NTR) of natural isolates of an epidemic echovirus 11' (prime). Arch Virol 2000; 145: 2575-2600.

119. Szendrői A., Delpeyroux F. et al. Manuscript in preparation from Institute Pasteur

120. Proudnikov D., Kirillov E., Chumakov K., Donlon J., Rezapkin G., Mirzabekov A. Analysis of mutations in oral poliovirus vaccine by hybridization with generic oligonucleotide microchips. Biologicals 2000; 28: 57-66.

121. Rotbart H.A. Enzymatic RNA amplification of the enteroviruses. J Clin Microbiol 1990; 28, 438-442.

122. Rotbart H.A., Sawyer M.H., Fast S., Lewinsky C., Murphy N., Keyser E.F., Spadoro J., Kao S.Y., Loeffelholz M. Diagnosis of enteroviral meningitis by using PCR with a colorimetric microwell detection assay. J Clin Microbiol 1994; 32: 2590-2592.

123. Halonen P., Rocha E., Hierholzer J., Holloway B., Hyypiä T., Hurskainen P., Pallansch M. Detection of enteroviruses and rhinoviruses in clinical specimens by PCR and liquid-phase hybridization. J Clin Microbiol 1995; 33: 648-653.

124. Zoll G.J., Melchers W.J., Kopecka H., Jambroes G., van der Poel H.J., Galama J.M. General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. J Clin Microbiol 1992; 30: 160-165.

125. Auvinen P., Hyypiä T. ECHO viruses include genetically distinct serotypes. J Gen Virol 1990; 71: 2133-2139.

126. Coller B.G., Chapman N.M., Beck M.A., Pallansch M.A., Gauntt C.J., Tracy S.M. Echovirus 22 is an atypical enterovirus. J Virol 1990; 64: 2692-2701.

127. Oberste M.S., Maher K., Pallansch M.A. Complete sequence of echovirus 23 and its relationship to echovirus 22 and other human enteroviruses. Virus Res 1998; 56: 217-223.

128. Strikas R.A., Anderson L.J., Parker R.A. Temporal and geographic patterns of isolates of nonpolio enterovirus in the United States, 1970-1983. J Infect Dis 1986; 153: 346-351.

129. Kessler H.H. Qualitative detection of herpes simplex virus DNA on the LightCycler. In: Meuer S, Wittwer CT and Nakagawara K, editors. Rapid cycle real-time PCR – methods and applications. Springer-Verlag, Heidelberg, Germany, 2001; 331-340.

130. Clewley J.P. The polymerase chain reaction, a review of the practical limitations for human immunodeficiency virus diagnosis. J Virol Methods 1989; 25: 179-188.

131. Berg J., Nagl V., Mühlbauer G., Stekel H. Single-tube two-round polymerase chain reaction using the LightCycler instrument. J Clin Virol 2001; 20: 71-75.

132. Landt O. Selection of hybridization probe sequences for use with the LightCycler. In: Meuer S, Wittwer CT and Nakagawara K, editors. Rapid cycle real-time PCR – methods and applications. Springer-Verlag, Heidelberg, Germany, 2001; 35-41.

133. Haushofer A.C., Kopty C., Hauer R., Brunner H., Halbmayer W-M. HCV genotypes and age distribution in patients of Vienna and surrounding areas. J Clin Virol 2001; 20: 41-47.

9. Original communications