

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

# **Postvaccination rotavirus surveillance in Hungary, 2007-2011**

by Brigitta László Antalné

Supervisors:

dr. Krisztián Bányai and dr. József Kónya



UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

Debrecen, 2013

# POSTVACCINATION ROTAVIRUS SURVEILLANCE IN HUNGARY, 2007-2011

By **Brigitta László Antalné**  
molecular biologist MSc (microbiologist)

Supervisors: dr. Krisztián Bányai and dr. József Kónya  
Doctoral School of Pharmaceutical Sciences, University of Debrecen

Head of the **Examination Committee:** dr. László Maródi, DSc  
Members of the Examination Committee: dr. Sándor Bíró, DSc  
dr. János Minárovits, DSc

The Examination takes place at Department of Infectious and Pediatric Immunology, Medical and Health Science Center, University of Debrecen, 11:00, 10. 04. 2013.

Head of the **Defense Committee:** dr. László Maródi, DSc  
Reviewers: dr. Attila Bácsi, PhD  
dr. Mária Takács, PhD  
Members of the Defense Committee: dr. Sándor Bíró, DSc  
dr. János Minárovits, DSc

The PhD Defense takes place at the Lecture Hall of Bldg. „A”, Department of Internal Medicine, Medical and Health Science Center, University of Debrecen, 15:00, 10. 04. 2013.

# INTRODUCTION

Acute gastroenteritis is a public health concern worldwide due to high morbidity and mortality. Among enteric viral pathogens group A rotaviruses (hGARV) have been identified as the most important etiological agents of acute diarrheal gastroenteritis in infants and children under 5 years of age. It has been observed globally that by the age of five years virtually all children have been infected by one or more rotaviruses. About 130-140 million new symptomatic primary rotavirus infections occur worldwide. Typical symptoms are abdominal pain, loss of appetite, vomiting, diarrhoea and fever, the most common complication is dehydration, which can also be life-threatening in severity. Group A rotaviruses are responsible for about 2 million hospitalizations and for an estimated 400,000-600,000 deaths annually in children less than 5 years of age, especially in the developing countries. In developed countries, including Hungary, economic burden related to hospital care costs is the main problem. In consideration of the global burden of mortality, hospitalization and clinical visits due to rotavirus infections, vaccination is the main strategy in control and prevention of the disease. After introduction of new generation of rotavirus vaccines, strain surveillance efforts have intensified worldwide.

Outer capsid antigens G and P are defined by VP7 and VP4 gene segments of double-stranded RNA genome. The surface proteins induce serotype-specific and cross-reactive neutralizing antibody production independently both in natural infection and after vaccination. Because these antibodies appear to play an important role in protection against subsequent severe rotavirus diseases and infections, they are the main targets of the vaccine development and that of the intensive surveillance studies throughout the world. Two live, oral, attenuated rotavirus vaccines have been licensed or already introduced from 2006 onward in several countries worldwide. Rotarix<sup>®</sup> (GlaxoSmithKline) is a monovalent G1P[8] rotavirus vaccine derived from a human rotavirus strain by serial passage in cell culture. RotaTeq<sup>®</sup> (Merck) is a pentavalent human-bovine (WC3) reassortant vaccine, with the five most common human type specificities, G1-G4 and P[8], expressed individually on the genetic backbone of a bovine rotavirus strain naturally attenuated for humans. The role of type-specific versus cross-protective immunity against rotavirus infection is still debated. Multiple doses of both vaccines confer protection against a wide diversity of human rotavirus strains, however, there are concerns regarding the effect of the vaccine strains on the dynamics of rotavirus epidemiology and the efficacy of the vaccines against emerging rotavirus strains completely

heterotypic to the vaccine strains. This concern has revived several international surveillance networks in parts of the world to assess this interplay between vaccine use and strain prevalence. European Rotavirus Network (EuroRotaNet) was launched with participation of Hungary in 2007 to monitor the geographical and temporal distribution of G- and P-types of rotaviruses in the postvaccine era and to estimate the impact of vaccination on strain prevalence and diversity.

## REVIEW OF THE LITERATURE

Rotaviruses are members of *Rotavirus* genus within the *Reoviridae* family and are responsible for an estimated about 600,000 deaths annually, primarily in developing countries. The virion encapsidates the 11 segments of double-stranded (ds)RNA. The genome encodes six structural (VP1- VP4, VP6, VP7) and six nonstructural proteins (NSP1-6). Rotaviruses are classified into groups or species (A-G) according to the antigenic and genetic features of the middle capsid layer protein, VP6. Out of the seven groups, only A-C are known to infect humans, from these groups A viruses are the most commonly associated with human infections. Other groups of rotaviruses have been detected so far only in animals.

The outer part of the triple-layered capsid is composed of two proteins encoded by VP4 and VP7 gene segments. VP4 defines P (protease-sensitive) and VP7 defines G (glycoprotein) antigens. Group A rotaviruses are usually characterized by these two outer capsid proteins. A binary classification system has been established on the basis of antigenic and genetic properties of surface antigens (G1P[8], G2P[4], G4P[6] etc). Recently a new classification system has been introduced which takes into account the genetic specificities of each of the 11 gene segments.

Up to the present, 27 G-types and 35 P-types have been described in humans and animals worldwide but human rotavirus strains have been shown to carry only 12 G and 15 P type specificities. Despite the great number of genotype combinations that can be theoretically derived from this large number of VP7 and VP4 genotypes, only a relatively few strains have been reported to have medical significance worldwide including genotypes G1P[8], G3P[8], G4P[8], G9P[8], and G2P[4]. The common G1, G3, G4 and G9 G-types are preferentially associated with P[8] genotype specificities (Wa-genogroup), whereas G2 type strains are most frequently associated with the P[4] genotype (DS-1-genogroup). Other uncommon antigen types and rare combinations of G- and P-types have been identified in different countries and many of them might be locally and temporally important. This spatiotemporal variation in strain prevalence contributes further to the regional strain diversity.

The great antigenic and genetic diversity of rotaviruses is owing to a complex evolutionary strategy. Accumulation of point mutations in the genome and reassortment of gene segments are known to be the most general occurrence to maintain the strain heterogeneity. It has been documented that mixed infections of the same cell with different types of rotaviruses can occur frequently, causing increased genomic reassortment.

Introduction of animal rotavirus genes to human population and gene rearrangements are another major sources of rotavirus diversity.

Rotaviruses spread between patients by faecal-oral route, commonly by contact of faecally contaminated hands, objects, food or water. Rotaviruses are shed in the faeces in a large amounts and already 10-100 particles are likely infectious. Clinical symptoms manifest after an incubation period of 12-48 hours. Diarrhoea and vomiting are the main clinical manifestations in infants and young children but fever, abdominal pain, lethargy and loss of appetite are also common symptoms. Natural rotavirus infection of adults is usually asymptomatic or can cause only mild symptoms. The severity of rotavirus infection varies from asymptomatic cases to fatal diseases due to the loss of electrolytes and water. The primary management of rotavirus gastroenteritis is the rehydration and restoration of electrolyte balance by oral or intravenous rehydration therapy.

Protection against rotavirus gastroenteritis among neonates and infants is mediated primarily by maternal antibodies transferred transplacentally or by breast-feeding. Maternal antibodies provide protection against severe rotavirus diseases up to 3-6 months of age. The first symptomatic rotavirus infection usually occurs between 12-24 months of age. The adaptive immunity develops after the first natural rotavirus infection. Protection against both reinfection and severe diarrhoea increased by sequential infections but lifelong protection will not develop. Rotavirus infection in adolescent and adult patients occurs mostly without or only with moderate symptoms. The outcome of infection can be severe or even fatal in elderly or immunodeficient patients.

The immune response to rotavirus infection is not completely understood. Cellular and humoral mechanisms contribute to clearance of rotavirus infection, although humoral immunity is considered to play a dominant role in protection. Neutralizing antibodies that recognize the VP4 and VP7 surface antigens are associated with protection from severe diseases. Type-specific and cross-reactive antibodies are produced during natural rotavirus infection.

The surface proteins, VP7 and VP4, elicit neutralizing antibodies independently *in vivo*, therefore they are the main targets of vaccine development. Two live, attenuated rotavirus vaccines have been licensed recently in several countries worldwide. Rotarix<sup>®</sup> (GlaxoSmithKline) is a monovalent human G1P[8] rotavirus vaccine. RotaTeq<sup>®</sup> (Merck) is a pentavalent human-bovine (WC3) reassortant vaccine consisted of the five most common types G1-G4 and P[8]. The rule of type-specific versus cross-protective immunity against rotavirus infection is still debated.

The introduction and use of recent vaccines raise several questions about rotavirus ecology which revived several international postvaccination surveillance networks all over the world. The European Rotavirus Network was launched with the participation of 18 European countries, including Hungary in 2007. The leading laboratory of the network is the Health Protection Agency in the United Kingdom. Rotavirus strain monitoring was conducted in Regional Laboratory of Virology, National Reference Laboratory of Gastroenteric Viruses, ÁNTSZ Regional Institute of State Public Health Service, Pécs and at the University of Debrecen, Medical and Health Science Center, Department of Medical Microbiology, Debrecen. Surveillance studies are necessary to document the emergence of new strains and identify the possible reassortment events between the vaccine and wild-type strains. Rotavirus strain monitoring allows assessing the impact of vaccines on rotavirus strain diversity and providing information on the evolution of strains not included in the vaccines.

## AIMS

- To extend the previous pre-vaccine era, involvement of additional geographical regions (eastern and western Hungarian counties) into our postvaccination surveillance study
- To determine the genotype specificity of VP4 and VP7 genes of circulating rotavirus strains by multiplex genotyping PCR assay
- To monitor the spatiotemporal distribution of detected rotavirus G and P types and to estimate the impact of vaccine use on strain diversity
- To identify the genotype specificity of non-typeable strains by nucleotide sequencing
- To evaluate the impact of rotavirus vaccines on genetic content of field strains by sequence and phylogenetic analysis of VP4 and VP7 genes of common strains presented in the vaccines
- To confirm the genotype specificity of unusual antigen combinations by sequence analysis of partial VP4 and/or VP7 genes
- To estimate the national vaccine coverage among infants in our country



## **MATERIALS AND METHODS**

### **Samples and patients data**

Rotavirus positive faecal samples were collected from inpatients and outpatients with acute gastroenteritis between January 2007 and December 2011. Stool samples were obtained from sporadic, community acquired cases of all age groups through a national network from collaborating laboratories in seven counties (Baranya, Borsod-Abaúj-Zemplén, Csongrád, Heves, Hajdú-Bihar, Szabolcs-Szatmár-Bereg and Vas counties) plus in the capital of Hungary. Viral antigen was detected from stool in different laboratories by various commercial methods: latex agglutination and immunochromatography tests performed according to manufacturers' recommendations were the primary detection methods. Rotavirus positive stool samples were transported to the Department of Medical Microbiology, Medical and Health Science Center, University of Debrecen and were stored on -20 °C.

Information on patients' age, sex, date of disease onset and sample collection, symptoms, geographical location and setting (community/hospital) were collected as baseline epidemiologic background data.

### **RNA extraction**

Stool samples were diluted in 10 mM Tris-HCl (pH 7.5) to obtain 10-20 % suspensions. Viral dsRNA was extracted from supernatant of fecal suspension using TriReagent or Roche High Pure Viral Nucleic Acid Kit following the manufacturers' instructions. The RNA was eluted in nuclease-free or DEPC-treated water and stored at -70 °C. PCR inhibitors were removed from extracted RNA by additional guanidine isothiocyanate and silica-matrix purification.

## **Genotyping by multiplex-nested PCR**

### **Reverse transcription and multiplex-nested PCR**

For RT-PCR 5 µl of extracted dsRNA was used. Complementary DNA was synthesized using AMV reverse transcriptase at 42 °C for 60 minutes in the presence of a cocktail of random-hexamers or VP4-F/con3 and VP7-F consensus primers specific for VP4 and VP7 genes of rotavirus.

Amplification and genotyping the cDNA was performed using a multiplex nested PCR assay. 5 µl of synthesized products in RT-PCR were used as template in the first round PCR. The primer pair VP4-F/VP4-R or Con2/Con3 was used for amplification of a region of the gene encoding VP4 and primer pair VP7-F/VP7-R was used to amplify a region of the gene encoding VP7. Genotyping was carried out using the first-round PCR product as a template in the presence of one of the consensus primers combination with genotype-specific primer mix. Type specific primers targeted to G1 to G4, G8 to G10, and G12 VP7 specificities and P[4], P[6] and P[8] to P[11] VP4 specificities were used in the specific primer mixes, respectively. Both first- and second-round PCR were performed in a total volume of 50 µl using Taq polymerase

### **Agarose gel electrophoresis**

Genotypes were determined according to the length of the amplicons after electrophoresis of second-round PCR products (in a 1.5 % agarose gel stained with ethidium-bromide, in buffer TBE [Tris/Borate/EDTA]). Our results were evaluated in a BioRad GelDoc gel documentation system.

### **VP6 specific PCR**

The samples, for which no amplified products in the two-step PCR could be detected, were subjected to a VP6 specific PCR to verify the presence of the rotavirus double-stranded RNA. The primer pair VP6-F/VP6-R was used for amplification of a region of VP6 gene

segment of group A rotaviruses. Samples negative for VP6 PCR were excluded from the study.

## **Sequencing**

### **Purification of PCR products**

VP4 and/or VP7 genes of samples untyped by RT-PCR genotyping and of selected G1P[8], G2, G3 and G4 strains were examined by sequence analysis. Amplified products in the first-round PCR using Con2/Con3 and VP7-F/VP7-R consensus primers were purified from the gel after electrophoresis (in 1 % preparative agarose gel stained with ethidium-bromide, in buffer TBE [Tris/Borate/EDTA]) with SV PCR Clean-up System. Purified PCR products were stored at – 20 °C

### **Sequence and phylogenetic analysis**

Direct sequencing was performed using the reverse PCR primers Con2 for VP4 gene segment and VP7-R for VP7 gene segment. The cycle sequencing reaction was carried out with the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Reaction Kit. PCR products after sequencing were purified with ethanol-sodium-acetate mixture (96% ethanol and 3M, pH 5.2 sodium-acetate). Sequence data were collected by means of an automated DNA analyzer ABI Prism 3100 Avant or ABI3500. Sequence chromatograms were cleaned using the BioEdit software. Multiple alignments were manually adjusted using the GeneDoc software. Phylogenetic analysis was performed with the MEGA5; neighbour-joining trees were generated using the p-distance model and bootstrap resampling was performed.

## RESULTS

During the study period, 2615 rotavirus positive stool samples were collected from patients under 5 years of age with acute gastroenteritis. Samples were collected in 7 different counties and in the capital city of Hungary. Of these, 2526 samples were available in sufficient quantity for further analysis. A total of 146 (5.8%) samples were omitted from the final analysis after they gave negative results in both nested multiplex genotyping PCR and the subsequent confirmatory VP6 gene specific PCR assay. In total, 2380 rotavirus strains were genotyped.

Baseline epidemiological information was available for 2213 (92.9%) specimens. Of these, 1859 (84.0%) and 354 (16.0%) samples were collected from inpatients and outpatients, respectively. Most samples (79.6%) were obtained from children under five years of age, with relative abundance of age 6 to 24 months (40.5%). A total of 465 samples (21.1%) were collected from adolescent and adult patients (max. age 91 years).

A seasonal pattern in rotavirus infections was obtained. The peak incidence of rotavirus infections was seen from February to May. The prevalence of common genotype combination in the peak months was not significantly different as in the other period of the years.

### Prevalence of rotavirus strains in Hungary

In total, both G and P types could be identified for 2297 (96.5%) strains, representing 17 different genotype combinations. The remainders were only partially typeable, including 41 strains for which the G type was determined and the P type remained untypeable and 41 strains for which only the P type could be determined.

In general, almost 91% of the 2380 strains belonged to one of the five globally common genotype combinations (G1P[8], 44.9%; G2P[4], 14.8%; G3P[8], 0.9%; G4P[8], 23.4%; G9P[8], 6.8%). Among the globally important human strains, only G3P[8] strains were not detected in each year. Less common genotypes were found in a small proportion (4%) of strains identified during the study period. These strains were unusual combinations of common human G and P types (G1P[4], 0.38%; G2P[8], 0.30%; G3P[4], 0.08%; and G9P[4], 0.08%;) and some other unusual G-P combinations (G1P[6], 0.04%; G2P[6], 0.04%; G3P[9],

0.38%;G4P[6], 0.38%;G6P[9] 0.30%;and G9P[6] 0.17%). Of interest, an increased activity of G12 strains (1.97%) resulted in higher detection rates for either G12P[8] or G12P[6] genotype than that of G3P[8] in some years.

## **Spatiotemporal distributions of common rotavirus strains**

Rotavirus positive samples were collected from eight different geographical regions. Five study sites provided samples in each consecutive season. Because different areas contributed with fluctuating amount of strains to the study in consecutive years, we depicted the spatiotemporal distribution of the five globally common rotavirus strains for the participating study sites. Differences in prevalence of these common rotavirus strains were found between different regions year by year. For example, G1P[8] was the most prevalent genotype in Northeast Hungary in 2007 and nearly in all geographical regions in the subsequent two years and in 2011 again in line with the increased prevalence of this genotype in this period. G2P[4] emerged to become predominant in Southwest Hungary in 2007 as well as in Northeast Hungary and the in capital area in 2010. A higher percentage of G4P[8] strains was observed in West Hungary and in the Budapest area in 2007 and in Southwest-, South- and East Hungary during 2010. The G9P[8] genotype was found at high prevalence in North-Eastern Hungary in 2008 and this strain was the second most prevalent genotype combination in Budapest and in central Hungary during 2011.

## **Sequence analysis of the VP7 and VP4 genes**

Sequence and phylogenetic analyses of partial region of VP4 and VP7 gene segments of rotaviruses were performed to define or verify the genotype of the nontypeable or the unusual strains. Results of verifications are included in prevalence data of G and P-types. On the other hand, phylogenetic analyses were performed to find the origin of rare genotype combinations and to know if vaccine strains could have interacted with field strains or vaccine strains may have reverted to become virulent and circulate in the community.

Shortly after the beginning of the 2007 surveillance year, four cases of G9P[6], rotavirus infections were identified in the south-western area of our network, representing the first documented occurrence of this strain in our surveillance area. Epidemiologic and anamnestic data suggested that these strains were newly introduced into our country, possibly from India. Phylogenetic analysis of the outer capsid genes of two selected strains was performed to confirm our hypothesis. Investigated regions of VP4 and VP7 genes of selected strains shared 100% sequence homology thus sequence analysis confirmed the epidemiologic linkage among the primary and the secondary cases. Sequence data for the Hungarian G9P[6] strains demonstrated no direct evolutionary relationship with cognate genes of former Hungarian strains with either G9 or P[6] specificities, but a close relationship was seen with human strains originating from South Asia. Lines of indirect evidence support our speculation that this new strain to Hungary may have been introduced into our surveillance area from India.

A subset of G1P[8] strains were selected from various years and study sites for nucleotide sequencing of the VP7 and VP4 genes. None of the 318 successfully sequenced strains were closely related to the G1 VP7 and P[8] VP4 genes of the pentavalent RotaTeq<sup>®</sup>. The maximum identity between circulating G1P[8] strains and corresponding specificities in the RotaTeq<sup>®</sup> was 93% for the VP7 gene and 94 % for the VP4 gene. In contrast, the VP7 gene of four community strains clustered together with the VP7 of strain 89-12, the parental strain of the Rotarix<sup>®</sup>. Furthermore 51 strains clustered together along with the Rotarix<sup>®</sup> strain in the VP4 phylogeny. However, none of these 55 strains detected during 2007-2009, shared both the P[8] VP4 and the G1 VP7 lineages with 89-12.

The VP7 gene of 32 rotavirus strains with G2, G3 and G4 genotype were partially sequenced and compared with the same sequences of RotaTeq<sup>®</sup> vaccine strains to measure the potential impact of vaccine usage on genetic content of local strains. The successfully sequenced strains were not closely related to the G2, G3 or G4 VP7 genes of the vaccine strains.

## **Methodological considerations**

The nucleotide sequencing of VP4 and/or VP7 gene segments of unusual strains were carried out to confirm our results. Results, obtained in genotyping PCR assay, were verified by sequencing, however, contradictions were found in some cases. One of this different results was found when we tried to confirm the VP7 specificity of some suspected G12P[9] strains. Sequencing of these viruses proved that the real VP7 genotype specificity of mistyped strains was G6. Our detailed examination showed a great nucleotide homology between G12 type specific primer and the primer binding site of G6 strains. We found only one nucleotide difference between the two sequences.

Another mistyping was the VP7 gene of a G8P[8] strain, whose G type proved to be G4 by nucleotide sequencing. Sequencing of a G2P[6] and a G4P[6] strain revealed the G12P[6] specificity of selected strains. Any remarkable sequence homology between genotyping primers and target sequences was not revealed as it was obtained in G6 strains, only few nucleotide identities were found.

## **Rotavirus vaccination in Hungary**

When vaccine sales data for each vaccine were combined with the number of annual live-birth we estimated that the overall vaccine coverage, among infants, increased from 4% to 18% between 2007 and 2010. Rotarix<sup>®</sup> was purchased more often (~90% relative use) in our country than the pentavalent RotaTeq<sup>®</sup> vaccine. Vaccine coverage in the capital city and in some of the Central-and West-Hungarian subregions was between 20 and 40% rarely achieves a remarkable 43-93% but the nationwide vaccine coverage among infants remained below 20%.

As information on potential vaccination status could not be collected during this study, we could not evaluate the potential impact of vaccination on severity of rotavirus infections in a particular geographical region.

## DISCUSSION

Rotavirus surveillance dates back to 1980s in Hungary, showing the dominating prevalence of G1-G4 strains in the early period. In the pre rotavirus vaccine era, two sentinel regions (Budapest and Baranya county) were included in the rotavirus surveillance in Hungary and approximately 7000 strains were sero- or genotyped during a 22 years period of time. One of our main aims was the extension of our study area to gain comprehensive information on genotype diversity of rotaviruses circulating in Hungary. Between 2007 and 2011 we genotyped over 2400 strains from eight study areas using the typing protocol recommended by the EuroRotaNet, the first European rotavirus strain surveillance network. Among the 2380 successfully genotyped rotaviruses, the most important strains were G1P[8], G2P[4] and G4P[8]. The relative significance of common G3P[8] strain is low in our country, the prevalence of this genotype remained under 1% in the study period. G9P[8] strains were detected as the fourth most common genotype in our study area. G9 strains emerged in mid-1990s and spread in several countries all over the world and became the fifth globally common genotype with increasing prevalence and medical importance. This antigen combination was found in 1998 for the first time in Hungary and after this it became the second most common genotype following G1P[8] strains. The prevalence of G9P[8] strains slightly decreased in the postvaccine era, but it was detected in every year and almost all of the geographic regions. Reports on emergence and increasing prevalence of G12 rotaviruses were published from several countries worldwide in the early 2000s. This genotype, mostly in combination with P[8] specificity, was detected in 2005 for the first time in our country but the prevalence of these strains was relatively low. The overall prevalence of G12P[6] and G12P[8] strains increased in the last five years and was higher than that of G3P[8] in the study period. The relative importance of G12P[6] genotype combination exceeded the prevalence of G12P[8] strains in the last few years. Our data indicated that G12 strains are the fifth most prevalent genotype circulating in Hungary. Increasing prevalence of these strains will require greater attention in the future.

In addition to common strains, we identified several rare or unusual antigen combinations in the study years. The overall prevalence of these uncommon strains is slightly more than 4%. The G9P[6] strain was detected from four cases of rotavirus gastroenteritis in our first surveillance year. The sequence and phylogenetic analysis proved the epidemiologic linkage of this four cases and confirmed our hypothesis as the G9P[6] strain was probably



introduced from India. The G9P[6] genotype is prevalent in certain areas of India, but in other parts of the world including Europe and Hungary this genotype is not the common cause of rotavirus diarrhoea. Evidence for subsequent epidemiologic capacity of this unusual antigen combination in Hungary was not obtained.

During 2006-2007 two rotavirus vaccines were licensed in Hungary, the pentavalent RotaTeq<sup>®</sup> and the monovalent Rotarix<sup>®</sup>, but the vaccines were not introduced in the national immunization program. When vaccine sales data for each vaccine was combined with the number of live-birth we estimated that the overall vaccine coverage, among infants, increased from 4% to 18% between 2007 and 2010. This low nationwide coverage is largely attributable to the exclusive private market availability and to the high costs of the vaccines. This low vaccine coverage is not suitable to elicit herd immunity or to observe the ecological and socio-economic impact of vaccination. In countries with high vaccine coverage it has been observed that G2P[4] strains are very common in countries where Rotarix<sup>®</sup> is used. In other regions where RotaTeq<sup>®</sup> is included in the vaccination schedule, the resurgence of G3P[8] strains has been documented. At present, no definitive conclusion can be made to declare, if such re-emergences of different strains in areas where different vaccines are used, are associated with vaccination or merely reflect the natural spatiotemporal fluctuation of common co-circulating strains. Consequently, it is challenging to assess if vaccine strains could have any impact on locally circulating strains in our country. However, concerns that vaccine use may influence local strain distribution remain. As information on potential vaccination status could not be collected during this study, we concluded that the only way to measure any possible effect of vaccine usage on local strain prevalence and diversity was to determine the genetic content of field strains through DNA sequencing and compare them to the vaccine strains. During the study period the molecular analysis of the genes encoding the surface antigens of G1P[8], G2, G3 and G4 field strains detected in different geographical regions and years was performed. Most of the selected field strains were G1P[8], as the Rotarix<sup>®</sup> vaccine was purchased more often in Hungary. The majority of sequenced viruses did not share the antigens with vaccine strains. However, 55 strains were closely related but not identical to the Rotarix<sup>®</sup> parental strain, indicating that the possibility of the vaccine strain circulating in the population, even if appeared unlikely, could not be excluded. Nonetheless, point mutations other than those indicating relatedness to the vaccine strain were present in these strains. The presence of these additional point mutations would suggest that these strains had been circulating in the population for some time and because of the low vaccine coverage the likelihood that vaccine strains could have reverted to wild type is low. The VP7 genes of

selected G2, G3 and G4 strains were not closely related to those of pentavalent vaccine strains.

The number of so far detected antigen combinations in Hungary exceed 20 including the 17 genotype combinations identified in our study period and the number of genotypes found in the prevaccine era. Reassortment of locally circulating strains, introduction of genotypes from other regions and transmission of animal rotaviruses or genes to human population each contribute to rotavirus strain diversity. The susceptible population may not have protective antibodies against neutralizing antigens of human-animal reassortant strains, therefore these reassortant viruses have the potential to infect and spread among humans. The lack of protective immunity of population against G9 and G12 strains of suspected porcine origin could have played an important role in global emergence and spread of these genotypes. The detection of G6, P[6] and P[9] genotypes in our study period raises the possibility of an animal source of this types. The sequence and phylogenetic analysis of partial or whole genome of rotaviruses may help to clarify the exact origin of these unusual strains.

The improved and standardized typing protocol of the European Rotavirus Network targeted the eight and six most common G type and P type specificities, respectively. In Hungary, all but few strains belonged to one of these common genotypes. The G6 VP7 specificity, which appears endemic in Hungary, is however, not targeted in the assay. We detected this rare genotype in a few cases when we tried to confirm the VP7 specificity of suspected G12P[9] strains by sequencing. Two additional G12 and one G4 strains were mistyped by PCR, although both of the adequate type specific primers were included in our assay. The control of accuracy and reliability of genotyping primers is crucial in the strain surveillance due to the relative rapid genetic changes of rotaviruses. Also, random selection of common and systematic confirmation of uncommon strains by nucleotide sequencing showed that the standardized protocol worked well in our hands, however, mistyping emphasizes the need for confirmatory assays (e.g. nucleotide sequencing) which are very useful when unexpected strains are identified with routine typing methods.

Enhanced nationwide rotavirus strain surveillance identified large antigenic diversity in Hungary after rotavirus vaccines became available for use during 2006 and 2007. The genotype specificities of field strains are partly or completely shared with strains included in both commercially available human rotavirus vaccines. Due to the low vaccine coverage we found no solid evidence for an impact of vaccine usage on natural strain dynamics and

evolution of rotaviruses. Future studies based on whole genome sequencing and more in-depth evolutionary analyses will certainly shed light on unanswered questions.

Surveillance data in EuroRotaNet database are available for pharmaceutical companies producing the rotavirus vaccines. Thanks for the international surveillance network we have the possibility to recognize the changes in rotavirus strain distribution and to answer the question whether the composition of recent vaccines will be needed to modify.

## SUMMARY

Vaccination is the main strategy to control severe dehydrating gastroenteritis caused by rotaviruses in early childhood. The introduction of new generation rotavirus vaccines, Rotarix<sup>®</sup> and RotaTeq<sup>®</sup>, has resulted in an intensification of strain surveillance worldwide to monitor strain prevalence across countries. An international strain surveillance network (EuroRotaNet) was launched in 2007 in Europe with the participation of Hungary. In the present study, the geographical and temporal distribution of human rotavirus G and P types and the impact of vaccine use on strain prevalence and diversity in Hungary are reported.

Rotavirus positive stool samples were collected from patients, mostly children <5 years of age with gastroenteritis in 8 geographic areas of Hungary 2007-2011. Viral RNA was amplified by multiplex genotyping RT-PCR assay, targeting the medically most important G and P types. Sequencing of the VP4 and VP7 genes was performed to monitor the genetic relatedness of rotavirus strains.

In total, 2380 strains were genotyped. During the five-year surveillance 17 different antigen combinations were identified with the dominating prevalence of genotype G1P[8] (44,9%) strains, followed by G4P[8] (23,4%), G2P[4] (14,8%) and G9P[8] (6,8%). The prevalence of common G3P[8] strain remained below 1% in our country. Additional rare genotypes were identified in a low percentage of samples (4.12%): G1P[4], G1P[6], G2P[6], G2P[8], G3P[4], G3P[9], G4P[6], G6P[9], G9P[4], G9P[6], G12P[6] and G12P[8]. The prevalence of common strains fluctuated both in the consecutive years and between geographical regions in the same rotavirus season. Sequence and phylogenetic analyses revealed evidence for the imported G9P[6] strains from India and some primer mistyping in our PCR assay (G6 and G12 strains). The sequence determination and comparison of VP4 and VP7 genes of selected field strains and vaccine strains did not prove direct genetic relatedness between them. However, G1 and P[8] types closely related but not identical with Rotarix<sup>®</sup> parental strains were determined, their vaccine origin was unlikely. Rotarix<sup>®</sup> was purchased more often, but the nationwide vaccine coverage among infants remained low (~ 18%).

Our data indicates that the antigen specificities of medically important rotavirus strains, identified in our country, are represented in the vaccines available in the pharmaceutical private market in Hungary. The intensification of vaccine use in the forthcoming years may help to decrease the socio-economic burden of rotavirus infections.

Register Number: DEENKÉTK/7/2013.

Item Number:

Subject: Ph.D. List of Publications

Candidate: Brigitta László Antalné

Neptun ID: DRS8UM

Doctoral School: Doctoral School of Pharmaceutical Sciences

### List of publications related to the dissertation

1. László, B., Kónya, J., Dandár, E., Deák, J., Farkas, Á., Gray, J., Grósz, G., Iturriza-Gomara, M., Jakab, F., Juhász, Á., Kisfali, P., Kovács, J., Lengyel, G., Martella, V., Melegh, B., Mészáros, J., Molnár, P., Nyúl, Z., Papp, H., Pátri, L., Puskás, E., Sántha, I., Schneider, F., Szomor, K., Tóth, A., Tóth, E., Szűcs, G., Bányai, K.: Surveillance of human rotaviruses in 2007-2011, Hungary: Exploring the genetic relatedness between vaccine and field strains.  
*J. Clin. Virol.* 55 (2), 140-146, 2012.  
DOI: <http://dx.doi.org/10.1016/j.jcv.2012.06.016>  
IF:3.969 (2011)
2. László, B., Nyúl, Z., Kisfali, P., Deák, J., Kovács, J., Kónya, J., Mészner, Z., Molnár, P., Pátri, L., Schneider, F., Tóth, A., Melegh, B., Iturriza-Gomara, M., Gray, J., Martella, V., Szűcs, G., Bányai, K.: First detection of P[6],G9 rotaviruses in Hungary: An imported strain from India?  
*J. Travel Med.* 16 (2), 141-143, 2009.  
DOI: <http://dx.doi.org/10.1111/j.1708-8305.2008.00293.x>  
IF:1.503
3. László B., Czellar E., Deák J., Juhász Á., Kovács J., Kónya J., Mészáros J., Mészner Z., Mihály I., Molnár P., Nyúl Z., Pátri L., Puskás E., Schneider F., Siffel C., Tóth A., Tóth E., Szűcs G., Bányai K.: Posztvakcinációs rotavírus-surveillance Magyarországon, 2007.  
*Orv. Hetil.* 150 (31), 1443-1450, 2009.  
DOI: <http://dx.doi.org/10.1556/OH.2009.28690>.



#### List of other publications

4. Bányai, K., **László, B.**, Duque, J., Steele, A.D., Nelson, E.A.S., Gentsch, J.R., Parashar, U.D.:  
Systematic review of regional and temporal trends in global rotavirus strain diversity in the pre  
rotavirus vaccine era: Insights for understanding the impact of rotavirus vaccination programs.  
*Vaccine*. 30 (Suppl. 1), 122-130, 2012.  
DOI: <http://dx.doi.org/10.1016/j.vaccine.2011.09.111>  
IF:3.766 (2011)
5. Iturriza-Gomara, M., Dallman, T., Bányai, K., Böttiger, B., Buesa, J., Diedrich, S., Fiore, L., Johansen,  
K., Koopmans, M., Korsun, N., Koukou, D., Kroneman, A., **László, B.**, Lappalainen, M.,  
Maunula, L., Marques, A.M., Matthinjssens, J., Midgley, S., Mladenova, Z., Nawaz, S., Poljsak-  
Priatelj, M., Pothier, P., Ruggeri, F.M., Sanchez-Fauquier, A., Steyer, A., Sidaraviciute-  
Ivaskeviciene, I., Syriopoulou, V., Tran, A.N., Usonis, V., van Ranst, M., de Rougemont, A.,  
Gray, J.: Rotavirus genotypes co-circulating in Europe between 2006 and 2009 as determined  
by EuroRotaNet, a pan-European collaborative strain surveillance network.  
*Epidemiol. Infect.* 139 (6), 895-909, 2011.  
DOI: <http://dx.doi.org/10.1017/S0950268810001810>  
IF:2.843
6. Iturriza-Gomara, M., Dallman, T., Bányai, K., Böttiger, B., Buesa, J., Diedrich, S., Fiore, L., Johansen,  
K., Korsun, N., Kroneman, A., Lappalainen, M., **László, B.**, Maunula, L., Matthinjssens, J.,  
Midgley, S., Mladenova, Z., Poljsak-Priatelj, M., Pothier, P., Ruggeri, F.M., Sanchez-Fauquier,  
A., Schreier, E., Steyer, A., Sidaraviciute-Ivaskeviciene, I., Tran, A.N., Usonis, V., van Ranst, M.,  
de Rougemont, A., Gray, J.: Rotavirus surveillance in europe, 2005-2008: Web-enabled  
reporting and real-time analysis of genotyping and epidemiological data.  
*J. Infect. Dis.* 200 (Suppl.1), S215-S221, 2009.  
DOI: <http://dx.doi.org/10.1086/605049>  
IF:5.865
7. Szalmás, A., Bánáti, F., Koroknai, A., **László, B.**, Fehér, E., Salamon, D., Gergely, L., Minárovits, J.,  
Kónya, J.: Lineage-specific silencing of human IL-10 gene expression by promoter methylation  
in cervical cancer cells.  
*Eur. J. Cancer*. 44 (7), 1030-1038, 2008.  
DOI: <http://dx.doi.org/10.1016/j.ejca.2008.02.046>  
IF:4.475



8. Bányai K., Deák J., Gray J., Iturriza-Gomara M., Kovács J., Kónya J., **László B.**, Martella V., Mészner Z., Mihály I., Molnár P., Nyúl Z., Pátri L., Schneider F., Tóth A., Szűcs G.: Hazai részvétellel megalakult az Európai Rotavírusörzs-figyelő Hálózat (EuroRotaNet).  
*Orv. Hetil.* 148 (43), 2043-2045, 2007.  
DOI: <http://dx.doi.org/10.1556/OH.2007.28159>

**Total IF: 22.421**

**Total IF (publications related to the dissertation): 5.472**

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

10 January, 2013



## LIST OF PRESENTATIONS RELATED TO THE DISSERTATION

**Brigitta László**, Judit Deák, Jim Gray, Miren Iturriza-Gomara, Julianna Kovács, József Kónya, Vito Martella, Zsófia Mészner, Molnár, Zoltán Nyúl, László Pátri, Ferenc Schneider, András Tóth, György Szűcs, Krisztián Bányai: Prevalence of Rotavirus strains in Hungary, 2007.

Annual Meeting of the Hungarian Society for Microbiology, October 15-17, 2008, Keszthely, Hungary

**Brigitta László**, Judit Deák, Jim Gray, Miren Iturriza-Gomara, Ágnes Juhász, Julianna Kovács, József Kónya, Vito Martella, Júlia Mészáros, Zsófia Mészner, Ilona Mihály, Péter Molnár, Zoltán Nyúl, László Pátri, Erzsébet Puskás, Ferenc Schneider, András Tóth, Erzsébet Tóth, György Szűcs, Krisztián Bányai: Occurrence of unusual and vaccine targeted rotavirus strains in a multicentric survey in Hungary.

Annual Meeting of the Hungarian Society for Microbiology, October 13-15, 2010, Keszthely, Hungary

**Brigitta László**, József Kónya, Judit Deák, Ferenc Jakab, Ágnes Juhász, Ildikó Sántha, Krisztián Bányai and the Hungarian Rotavirus Surveillance Network: Surveillance of human rotaviruses in 2007-2011, Hungary.

Annual Meeting of the Hungarian Society for Microbiology, October 24-26, 2012, Keszthely, Hungary

Papp Hajnalka, **László Brigitta**, Bányai Krisztián: Rotavírus eredetű zoonózisok. OEK Virologiai Napok, 2012. Budapest

## LIST OF POSTERS RELATED TO THE DISSERTATION

Bányai K, Bogdán Á, **László B**, Domonkos G, Molnár P, Mihály I, Kónya J, Martella V, Szűcs Gy.: The last 3 years of national rotavirus strain surveillance activity in the prevaccine era (2003-2006): first detection of serotype G8 and G12 rotaviruses in Hungary.

15th International Congress of the Hungarian Society for Microbiology, July 18-20, 2007, Budapest, Hungary

**László B**, Deák J, Gray J, Iturriza-Gomara M, Kovács J, Kónya J, Martella V, Mészner Z, Molnár P, Nyúl Z, Pátri L, Schneider F, Tóth A, Szűcs Gy, Bányai K.: First year of the post-vaccination rotavirus surveillance in Hungary.

8th International Rotavirus Symposium, 2008, Istanbul, Turkey

**László B**, Czellár E, Deák J, Gray J, Iturriza-Gomara M, Juhász Á, Kovács J, Kónya J, Martella V, Mészáros J, Mészner Zs, Mihály I, Molnár P, Nyúl Z, Pátri L, Puskás E, Schneider F, Tóth A, Tóth E, Szűcs Gy, Bányai K.: Rotavirus strains in Hungary, 2007-2008. 2nd Central European Forum for Microbiology, October 7-9, 2009, Keszthely, Hungary

**Brigitta László**, Hajnalka Papp, Eszter Dandár, Judit Deák, Jim Gray, Miren Iturriza-Gomara, Ferenc Jakab, Ágnes Juhász, Julianna Kovács, József Kónya, György Lengyel, Vito Martella, Júlia Mészáros, Zsófia Mészner, Ilona Mihály, Péter Molnár, Zoltán Nyúl, László



Pátri, Erzsébet Puskás, Ferenc Schneider, András Tóth, Erzsébet Tóth, György Szűcs, Krisztián Bányai: Prevalence of human rotavirus strains in Hungary, 2006-2011. Fourth European Rotavirus Biology Meeting, October 2-5, 2011, Altafiumara - Santa Trada Di Cannitello, Villa San Giovanni (RC) – Italy

**Brigitta László**, Hajnalka Papp, Eszter Dandár, Judit Deák, Jim Gray, Miren Iturriza-Gomara, Ferenc Jakab, Ágnes Juhász, Julianna Kovács, József Kónya, György Lengyel, Vito Martella, Júlia Mészáros, Zsófia Mészner, Ilona Mihály, Péter Molnár, Zoltán Nyúl, László Pátri, Erzsébet Puskás, Ferenc Schneider, András Tóth, Erzsébet Tóth, György Szűcs, Krisztián Bányai: Emerging human rotavirus strains, 2006-2010, Hungary. International Meeting on Emerging Diseases and Surveillance, Vienna, Austria • February 4–7, 2011

**László Brigitta**, Deák Judit, Jim Gray, Miren Iturriza-Gomara, Juhász Ágnes, Kovács Julianna, Kónya József, Vito Martella, Mészáros Júlia, Mészner Zsófia, Mihály Ilona, Molnár Péter, Nyúl Zoltán, Pátri László, Puskás Erzsébet, Schneider Ferenc, Tóth András, Tóth Erzsébet, Szűcs György, Bányai Krisztián: Rotavírus-törzsek molekuláris epidemiológiája a poszt-vakcinációs időszakban Magyarországon. A Magyar Infektológiai és Klinikai Mikrobiológiai Társaság 38. Kongresszusa, Debrecen, 2010. szeptember 30. - október 2.