MOLECULAR GENETIC EXAMINATION OF PARALYTIC TYPE 3 POLIOVIRUS ISOLATES AND MOLECULAR EPIDEMIOLOGY OF NON-POLIO ENTEROVIRUSES IN HUNGARY

Doctoral Thesis

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INTRODUCTION

The paralytic poliomyelitis *(poliomyelitis anterior acuta)* had been one of the most dreaded diseases of mankind, which had been caused by the polioviruses belonging to the virus family *Picornaviridae*. In order to prevent the epidemic occurrence of paralytic poliomyelitis, oral live monovalent poliomyelitis vaccine (mOPV) was used from 1959 to 1992 in Hungary. The last intrinsic disease occurred in 1969 in the country and the last imported infection was registered in 1972. Later it became clear, however, that the attenuated vaccine viruses may also cause *poliomyelitis* at a very low frequency. According to the calculations, the risk of vaccination-associated paralyses was significantly higher between 1961-1991 following the administration of mOPV type 3 than after the administration of mOPV types 1 and 2.

Nowadays the wild poliovirus types 1 and 3 are endemic in Africa, South-Eastern Asia and in India, Pakistan and Afghanistan. Poliovirus type 2 has not caused paralysis since 4 years, however, the vaccine derived paralytic poliomyelitis strains are still circulating among the population of Nigeria (cVDPV). The replacement of the trivalent OPV and reintroduction of the monovalent OPV (mOPV) has been decided by WHO because of financial reasons. Based on Hungarian experiences on the long-term use of mOPV type 3, the WHO recommended the molecular genetic examination of paralytic type 3 virus isolated from 1960 to 1967 in Hungary.

Seven countrywide enterovirus epidemics occurred in Hungary since the introduction of isolation methodology and identification. In addition to two poliovirus epidemics in 1957 and 1958, coxsackievirus B3 has caused a nation-wide epidemic of the Bornholm disease (*pleurodynia*) in 1958. The number of medically documented diseases reached 40,000. The last epidemic of hand, foot and mouth disease (HFMD) caused by coxsackievirus A 16 was reported in 1976. Enterovirus 71 caused 1550 clinical *serous meningitis* and *encephalitis* cases, of which 30 had lethal outcome in 1978. Echovirus 11' (prime) strain caused hemorrhagic hepatitis in 386 babies, of which 13 died in 1989. Recently, non-polio enteroviruses caused serious epidemics in Far-East Asian countries.

The classical methodology used in the routine diagnostic virology for the identification of enteroviruses (i.e. isolation in cell cultures and suckling mice) were found to be inappropriate for virus typing. In the frames of the present work, the methodology of the molecular typing was used to identify viruses detected from 2000 to 2008 in Hungary, in

order to reveal molecular epidemiology and the role of genetic variability of viruses in the pathogenesis of different illnesses.

STUDY OBJECTIVES

1. ***Molecular examination of poliovirus 3 isolates causing flaccid paralysis:***
   - 1.1. To seek the virological and clinical data associated with the archived poliovirus type 3 isolates;
   - 1.2. Are the paralytic type 3 isolates from 1960 to 1967 of wild type origin or vaccine derived poliovirus strains (VDPV)?
   - 1.3. In case the etiological agent of the paralysis has been the poliovirus, it has to be determined whether it has Sabin like nature or vaccine derived character (VDPV);
   - 1.4. In case the paralytic disease has been responsible for the disease, the molecular mechanism of the recovery of the neurovirulent character has to be detected.
2. ***Molecular characterization and epidemiology of non-polio enteroviruses in Hungary:***
   - 2.1. Introduction of the methodology of the molecular typing in the routine diagnostics of enteroviruses;
   - 2.2. Molecular typing of the enterovirus positive samples detected from 2000 to 2008;
   - 2.3. Molecular epidemiological characterization of the serotypes identified;
   - 2.4. Comparison of recently identified serotypes with the molecular properties of previous viruses causing epidemics with using phylogenetic analysis.

MATERIALS AND METHODS

Poliovirus samples obtained from vaccine-associated paralytic illnesses

Altogether 18 poliovirus type 3 isolates were obtained from 1960 to 1967 from paralytic poliomyelitis patients in spite of the nation-wide application of monovalent OPV. Based on the archived documents, the origin of the samples could be identified. The samples have been taken from 15 children suffering from poliomyelitis between 5 months and 4.5 years of age (median 1.5 years).

Non-polio enterovirus samples

From the diagnostic samples submitted to the Department for Virus Diagnostics of the National Center for Epidemiology (fecal samples, throat swabs and vesicular fluid) a group was selected, which were found positive using the universal enterovirus PCR detecting the 5'-NTR sequences. The sampling occurred between 2000 - 2008. The 93 samples were from 79

patients of 6 to 33 years of age (median 8.25 years). Enterovirus sequences could be detected in 33 samples. The majority of the patients had neurologic symptoms: *aseptic meningitis, encephalitis* or non-neurological illnesses: i.e. hand-foot and mouth disease (HFMD) or *herpangina*, but sometimes atypical symptoms occurred, too.

Sample preparation Non-sterile samples were processed according to WHO protocol (WHO, 2004).

Virus isolation For molecular characterization, isolates (second or third passage in primary monkey kidney cells) were passaged at 37˚C once in L20B monolayers and again in RD cells (human rhabdomyosarcoma ATCC CCL 136) to produce high-titer cultures.

Nucleic acid extraction RNA extraction was done by phenol-chloroform method and by silica-membrane based method with using QIAamp Viral RNA Mini Kit (Qiagen GmBH, Germany), according to the manufacturer's instructions.

RT-PCR analysis The intratypic serodifferentiation of poliovirus isolates was done by RT-PCR kit {WHO, 2004). Primer sequences and conditions for 5'-NTR and capsid coding region RT-PCR described in detail by *Kapusinszky et al., 2009*. Type 3 vaccine-related isolates were further screened for recombination in multiplex RT-PCR assays using primers specifically targeting sequence intervals in the P2 and P3 regions characteristic for each Sabin strain. Detection of non-polio enteroviruses and sequencing of 5'-NTR and VP1 region was reported by *Kapusinszky et al., 2010.*

Nucleotide sequencing PCR products were purified with PCR Clean up-M Kit (Viogene, Sunnyvale, CA). The sequencing reaction was done with Big DYEnamic ET dye terminator kit (Ammersham Pharmacia, Germany) according to the manufacturer's instruction. The electrophoresis was carried out on MegaBACE 1000 Sequence Analyzer. Sequences were corrected by *Finch TV v.1.4.0* program and subjected to nucleotide-nucleotide BLAST analysis

Phylogenetic analysis. Alignment of all sequences was done by *CLUSTALW v. 2.0.* Phylogenetic trees were constructed in 5'-NTR and VP1 genetic regions for all sequences with neighbor-joining algorithm implemented in *MEGA v.4.0.2.* Bootstrap analyses were performed on 1.000 replicates to generate confidence for groupings.

Amino acid sequence analysis. Translation of nucleotide sequences to amino acid level was done by using *Transseq* program.

Prediction of RNA secondary structure. Prediction of secondary structure was performed using *MFOLD* program.

SUMMARY OF THE EXPERIMENTAL DATA

Eighteen poliovirus type 3 isolates, isolated between 1960 and 1967 from paralytic patients in Hungary, were examined. The archived viruses were obtained from 15 VAPP patients. The objective of the examinations was based on the observation that the VAPP risk had been much higher after administration of Sabin type 3 vaccine, than that for types 1 and 2, but the molecular technologies had not been available at the time of vaccination.

First, it has been confirmed that all isolates were of vaccine (**Sabin-like**) origin. The nucleotide sequence of the 5'-NTR region of all genomes harbored one single point mutation (**U472C** within the hairpin-loop V. of the IRES element) responsible for the reversion of OPV3 viruses to wild type phenotype (**Figure 1**).

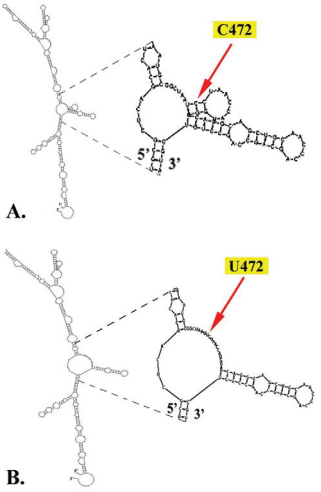

Figure 1. RNA secondary structure at 5'-noncoding region (385 nt) for all archived isolates (**A.**) in comparison with Sabin-3 reference strain AY184221 (**B.**). *Position in the genome: 190-575 nt.*

The nucleotide sequencing confirmed that the amino acid change **A54V** within the **VP1** capsomere protein was also present. It was shown that it could change the thermosensitive properties of poliovirus 3 vaccine strain together with the U472C mutation.

An A54T replacement could be detected in some thermoresistant isolates, suggesting similar effect to the heat resistance. The number of mutations did not exceed 1% within the VP1 region, therefore, all isolates were classified as Sabin-like. Isolates can be grouped into the VDPV cluster in case the mutation rate is above 1%, indicating prolonged virus shedding.

One of eighteen isolates was shown to be a recombinant. The recombinant partner was Sabin type 1 within the 3D polymerase coding region. This is a first detection of Sabin 3 recombinant from VAPP patient.

In connection with the re-evaluation of the findings obtained in connection with the mOPV vaccination campaigns, the number of children shedding non-polio enteroviruses was reduced by a factor of 4 during the 5 months of the campaigns. One has to be aware, that upon the introduction of inactivated poliovirus vaccines the epidemiological importance of non-polio enteroviruses will increase.

Molecular methodology was used between 2000 and 2008 for the detection of non-polio enteroviruses in order to detect uncultivable viruses. The presence of enteroviruses was detected using 5'-NTR nested RT-PCR. The typing of the viruses was done by nucleotide sequencing of conservative 5'-NTR and the variable VP1 region being in good correlation with serotypes. The typing proved to be successful in the case of 29 samples.

Coxsackievirus A16 was present in 12 samples. CV-A16 caused epidemics of hand-foot and mouth disease in 2000 and 2008 in Hungary. Sporadic infections were identified in almost every year causing neurologic illnesses of children under 5 years of age. The attack rate in different age groups was about 40% in the outbreak in 2008. We suppose an association between the clinical manifestation and the secondary structure of the 5'-NTR regions of CV-A16. We also presume that the differences between the secondary structures of virus RNAs were probably caused by mutations or recombinations that may influence the pathogenic properties of the viruses.

Enterovirus 71 was detected in 6 samples. EV71 caused neurological illnesses in Hungary. EV71 isolates detected between 2000 and 2008 were defined taxonomically as genotypes C1 and C4. The enterovirus 71 with high neurovirulence isolated in 1978 in Hungary had been classified as genotype B1 (**Figure 2**). Nucleotide sequence data revealed that 5'-NTR secondary structures belonged to 3 three different genotypes: C1, C4 and B1 respectively.

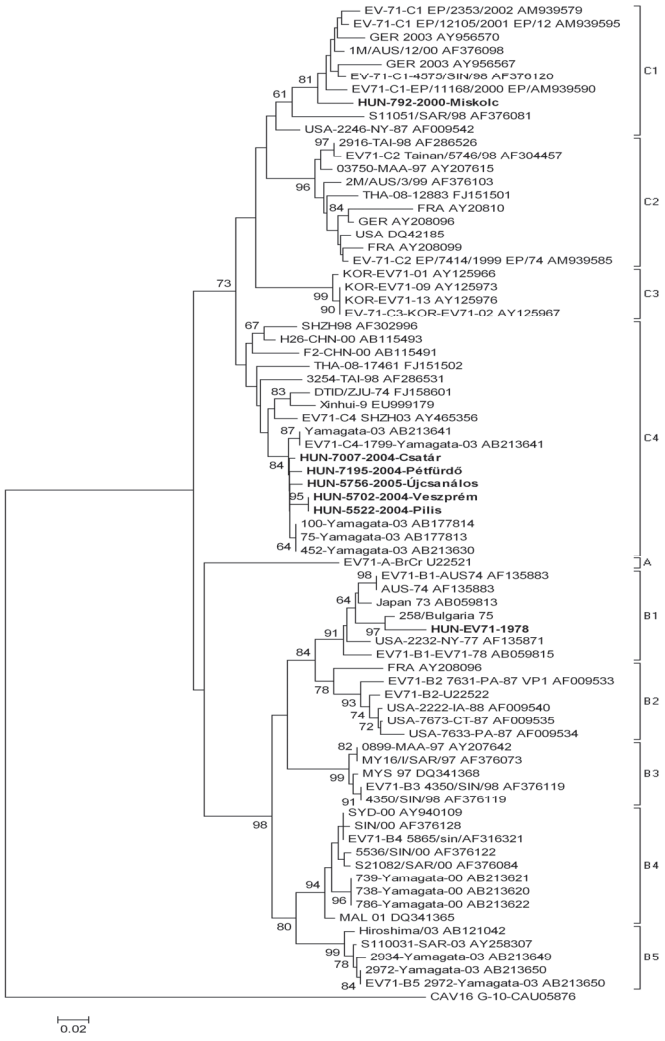

Figure 2. Phylogenetic relationships of enterovirus 71. *The tree was generated by using 255 nt fragments of the VP1. Bootstrap values >60% are shown in the branch nodes. The scale bar shows a genetic distance of 0.02. Coxsackievirus A16 G-10 (CAU05876) was used as an outgroup.*

Echovirus type 30 was detected from 13 samples, this is one of the most common non-polio enterovirus serotype which could cause *aseptic meningitis* in children around 10 years of age. Waves of echovirus 30 activity are associated with distinct new genomic lineages, which usually replace previously circulating ones. Four different echovirus 30 lineages causing neurologic illnesses were detected between 2000 and 2008 in Hungary (**Figure 3**).

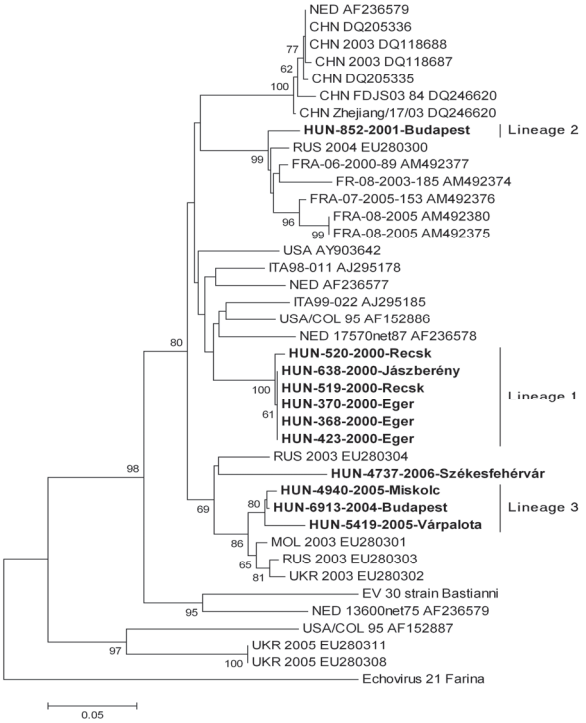

Figure 3. Phylogenetic relationships of echovirus 30. *The tree was generated by using 303 nt fragments of the VP1. Bootstrap values >60% are shown in the branch nodes. The scale bar shows a genetic distance of 0.05. Echovirus 21 Farina (AY302547) prototype strain was used as an outgroup.*

In conclusion, the results of molecular typing experiments performed by the two genetic regions of non-polio enteroviruses were similar regarding virus serotype. However, the sequence analysis targeting the variable VP1 region, which shows close relationship with virus serotype, was found to be more suitable for phylogenetic studies than 5'-NTR.

THE THESIS HAS BEEN PREPARED BASED ON THE PUBLICATIONS LISTED BELOW

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