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**MOLECULAR DETECTION OF NOROVIRUSES - POLIOVIRUSES IN THE ENVIRONMENT. IDENTIFICATION OF GENETIC RECOMBINATIONS AND CORRELATION WITH THE REPLICATION EFFICIENCY OF VIRAL STRAINS.**

## ABSTRACT

Polioviruses are members of the enterovirus genus, belonging to the Picornaviridae family. They are the causative agents of poliomyelitis, a paralytic and sometimes fatal disease in humans. The number of poliomyelitis cases caused by wild poliovirus infections has been drastically reduced by the extensive use of two available vaccines: the inactivated poliovirus vaccine (IPV) and the oral poliovirus vaccine (OPV). Despite the importance of OPV in the reduction of poliomyelitis cases, one of the disadvantages associated with this vaccine is the rare occurrence of vaccine-associated paralytic poliomyelitis (VAPP) in vaccinees or their healthy contacts through the accumulation of mutations and/or recombination in Sabin strains genome.

Thirteen clinical isolates originating from healthy vaccinees and VAPP cases were initially investigated in order to identify genomic modifications in 5' non-coding region (5'-NCR) and VP1 genomic regions. The analysis of samples was conducted by RT PCR, RFLP, sequencing and bioinformatics analysis. All clinical isolates were characterized as OPV-like viruses. Our results showed that analysis of 5'-NCR and VP1 regions of Poliovirus Sabin strains is important in order to identify mutations that increase neurovirulence and may eventually lead to the emergence of VAPP cases.

Mutations at specific sites of the genome and recombination between Sabin strains may result in the loss of the attenuated phenotype of OPV strains and the acquisition of traits characteristic of wild polioviruses, such as increased neurovirulence and loss of temperature sensitivity. In this view, we determined the phenotypic traits such as temperature sensitivity and growth kinetics of fourteen OPV isolates (recombinant and non-recombinant). The growth phenotype of each isolate as well as of Sabin vaccine strains in Hep2 cell line at two different temperatures (37$^{o}$C and 40$^{o}$C) was evaluated using two different assays, RCT test (Reproductive Capacity at different Temperatures) and one-step growth curve analysis. Moreover, the nucleotide and amino acid positions in the genomes of the isolates that have

been identified as being involved in the attenuated and thermo sensitive phenotype of Sabin vaccine strains were investigated. Mutations that result in loss of the attenuated and thermo sensitive phenotype of Sabin vaccine strains were identified in 5'-NCR, VP1 and 2C-3'NCR genomic regions of all isolates. Both mutations and recombination events correlated well with the reverted phenotypic traits of OPV-derivatives. The results showed that in Sabin-2 isolates with recombination type S2/S1, the mutation in residue 143 of VP1 capsid protein favours considerably the viral growth phenotype and may lead to the reversion towards neurovirulence. The substitution in residue 143 has been frequently observed in Sabin-2 strains isolated from VAPP cases. In isolates of Sabin 3 genotype, the recombination of Sabin 3 vaccine strain with Sabin 1 or Sabin 2 and the acquisition of genome structure S3/S1 or S3/S2 favour the growth phenotype and the reversion towards neurovirulence. This observation is in accordance with the fact that S3/SX recombinants have been frequently isolated from VAPP cases. Moreover, the presence of Sabin 1 vaccine strain as a 3' partner in bi-recombinants vaccine derived polioviruses (S3/S2/S1) favors the growth phenotype and may lead to the reversion towards neurovirulence. This is in accordance with the fact that recombinants Sabin-2 and Sabin-3 derivatives have been frequently isolated from VAPP cases while none of the Sabin-1 derivatives isolated from VAPP cases were found to be recombinants. The above may correlate with the increased fitness of SX/S1 recombinants than that of the S1/SX recombinants. In contrast, the bi-recombinants vaccine derived polioviruses S3/S2/S3 and S1/S3/S2 showed growth kinetics similar to that of Sabin vaccine strains. In the post-eradication era of wild polioviruses, the identification and the characterization (genomic and phenotypic) of vaccine-derived polioviruses become increasingly important in order to prevent cases or even outbreaks of paralytic poliomyelitis caused by neurovirulent strains.

Moreover, the serological status of southern Greek population of age groups 1-10, 11-20, 21-30 and 31-40 against Sabin vaccine strains and a collection of 15 recombinant and 4 non-recombinant Poliovirus vaccine strains was determined. No significant differences in NT titers of age group 1-40 were observed for the majority of OPV-derivatives in comparison with Sabin vaccine strains. However, significant lower NT titers were observed against two Sabin-1 derivatives, the first with recombination type S1/S3/S2 and the second non-recombinant, in comparison with Sabin 1 vaccine strain. For all three poliovirus types, the highest NT titers were observed in age group 1-10 while the lowest NT titer was observed in age group 21-30 against Poliovirus type 3. The serological status of the population of southern Greece against polioviruses is better for types 1 and 2 than type 3. The presence of the lowest NT

titer in age group 21-30 against Poliovirus type 3 suggests the need for a booster dose of monovalent Sabin 3 vaccine to ensure personal and herd immunity. As the preponderance of countries certified to be polio-free has switched from OPV to IPV and taking into consideration that IPV does not induce the same immunity as OPV, the need for immunological studies in all age groups is urgent in order to avoid epidemics due to the circulation of highly-evolved OPV-derivatives and the importation of wild polioviruses from endemic countries.

A multiplex RT-PCR screening analysis for identifying the predominant recombination types in 2C and 3D non-structural regions of vaccine-derived poliovirus strains was also developed in this thesis. In particular, two multiplex PCR were used for the identification of the predominant recombination types S3/SX (SX: S2 or S1) and S2/SX (SX: S3 or S1) in 2C and 3D, respectively, genomic regions of vaccine-derived poliovirus strains. To test the robustness of the proposed RT-PCR screening analysis, eleven recombinant vaccine-derived polioviruses that were characterized previously by sequencing by our group, in addition to three recently identified recombinant environmental isolates were assayed. Although the most definitive characterization of VDPVs is by genomic sequencing, in this study we describe a new, inexpensive and broadly applicable RT-PCR assay for the identification of the predominant recombination types in 2C and 3D genomic regions of VDPVs, that can be readily implemented in laboratories lacking sequencing facilities as a first approach for the early detection of VDPVs.

Finally, the detection of noroviruses which account for >90% of the reported outbreaks of non-bacterial gastroenteritis, was conducted in clinical and environmental samples. The detection of norovirus strains (in two out of eight clinical samples) was followed by sequencing of these genomic regions that allowed the designing of new primer pairs in order to sequence the whole genome. The sequencing of a genomic region, which includes the 3' end of ORF1, the whole ORF2 and the 5' end of ORF3 (nucleotides 4460-7421), of a viral strain (A6) isolated from stools of a patient with gastroenteritis was conducted. Moreover, norovirus strains were detected in three out of eight environmental samples originating from the sewage treatment plant of cities of Larissa, Trikala and Ioannina. Despite the use of a wide range of primer pairs (new and already used in previous studies), the sequencing of a small genomic region (280-700 bp) was achieved in each of the three environmental viral strains and in the second clinical viral strain. Finally phylogenetic analysis of the clinical strain A6 was conducted. Three phylogenetic trees for each of the three open reading frames (ORF1, ORF2 and ORF3) were constructed. In all three phylogenetic trees the strain A6 was clustered with viral

strains (deposited in GeneBank) of genotype GII. It is noteworthy the fact that the strain A6 was clustered with strains originating from different geographic regions in the phylogenetic trees of ORF1 and ORF2. In particular, the strain A6 was clustered with strains isolated from the western Sweden and the Japan in the phylogenetic trees of ORF1 and ORF2, respectively. This fact indicates the presence of a recombination event in the junction between ORF1 and ORF2 genomic regions which is in accordance with the observation that the noroviruses usually recombine in this region. Phylogenetic trees were not constructed for the other norovirus strains isolated in this study due to the small genomic region sequenced. The sequencing analysis of noroviruses isolates is of importance in order to accomplish phylogenetic and epidemiological studies and define the precise position of recombination events.