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Abstract

Attenuated strains of Sabin poliovirus vaccine replicate in the human gut and in rare cases may cause vaccine-associated paralytic poliomyelitis (VAPP). The genetic instability of Sabin strains constitutes one of the main causes of VAPP, a disease that is most frequently associated with type 3 and type 2 Sabin strains and more rarely with type 1 Sabin strains. In the present study, the growth phenotype of eight OPV isolates (two non-recombinants and six recombinants) as well as of Sabin vaccine strains was evaluated using two different assays, Reproductive Capacity at different Temperatures test (RCT test) and one-step growth curve test in Hep2 cells at two different temperatures (37^{o}C and 40^{o}C). The growth phenotype of isolates was correlated with genomic modifications in order to identify the determinants and mechanisms of reversion towards neurovirulence. All the recombinant OPV isolates showed a thermoresistant phenotype in RCT test. Moreover, both recombinant Sabin-3 isolates showed significantly higher viral yield than Sabin 3 vaccine strain at 37^{o}C and 40^{o}C in one-step growth curve test. All the OPV isolates displayed mutations at specific sites of the viral genome, which are associated with the attenuated and temperature sensitive phenotype of Sabin strains. The results showed that both mutations and recombination events could affect the phenotype traits of Sabin derivatives and may lead to the reversion of vaccinal strains to neurovirulent ones. The use of phenotypic markers along with the genomic analysis may shed additional light in the molecular determinants of the reversed neurovirulent phenotype of Sabin derivatives.

Keywords: OPV isolates; mutations; recombination; temperature sensitivity; growth kinetic analysis

Introduction

Poliovirus, the causal agent of poliomyelitis, is a member of the enterovirus genus of the Picornaviridae family and exists as three immunologically defined serotypes (P1, P2 and P3). The poliovirus is a nonenveloped virus composed of a 7.5-Kb positive sense single-stranded polyadenylated RNA genome surrounded by an icosahedral capsid [1]. The capsid is made of 60 protomers, each containing a single copy of the four structural capsid proteins (VP1 to VP4). The viral RNA contains a long open reading frame flanked by 5' and 3' noncoding regions (5'-NCR and 3'-NCR, respectively) involved in viral replication and translation. The predicted secondary structure of the 5'-NCR defines six domains (numbered from I to VI) corresponding to highly conserved structures formed by stems and loops [2, 3]. Domains II, IV, and V and part of domain VI contribute to the internal ribosomal entry site which allows cap-independent initiation of translation of the viral genome [4]. Structural and nonstructural viral proteins are coded by a single polypeptide precursor which is cleaved by viral proteases.

Since 1960, poliomyelitis has been effectively controlled by the use of inactivated or live attenuated vaccines. The Sabin live oral poliovirus vaccine (OPV) is constituted of attenuated strains of each of the three serotypes (Sabin 1, 2, and 3) which have been selected by numerous passages of wild-type strains in monkey tissues in vivo and in vitro [5]. The Sabin strains are temperature sensitive (ts) and the ts phenotype correlates with the attenuated phenotype [6-8]. The molecular determinants of attenuation and temperature sensitivity have been intensively studied to elucidate the mechanisms involved and to improve the safety of Sabin vaccine strains. The genome of Sabin 1 differs from that of its parental virulent Mahoney virus by 54 point mutations.

Molecular determinants of the attenuation and temperature sensitivity of Sabin 1 have been found in the 5'-NCR and 3'-NCR of the genome, as well as in the regions encoding VP1, VP3 and VP4 capsid proteins and the RNA polymerase (3D) [9-15]. For Sabin types 2 and 3, the vaccine strains differ from their progenitors by 23 and 11 mutations, respectively, and there is evidence in both cases that attenuation is determined by just 2 or 3 of these mutations. One substitution in the 5'-NCR of the genome and another that changes an amino acid residue in a capsid protein (VP1 in Sabin 2 and VP3 in Sabin 3) are the major determinants of attenuation and temperature sensitivity [7, 8, 15-22].

Undoubtedly, the use of OPV in mass vaccinations has resulted in drastic reductions in the number of poliomyelitis cases due to infections with wild-type polioviruses from an estimated 350,000 cases in over 125 endemic countries in 1988 to just 1,310 cases in four countries in 2007. However, in rare cases (1 case per 750,000 primary vaccinees), OPV strains have been implicated in vaccine-associated paralytic poliomyelitis (VAPP) [23]. The underlying cause of VAPP is the genetic instability of OPV strains. During multiplication in the human intestine, mutations at specific sites of the genome are associated with the attenuated and temperature sensitive phenotype of Sabin strains 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 [24-26]. Moreover, changes are frequently observed in antigenic properties of OPV derivatives representing a selection of viral variants that are less prone to be neutralized by human antibodies [27].

The aim of the present study was to correlate the reverted phenotypic traits of OPV isolates such as temperature sensitivity and growth kinetics with mutations and recombination events on the viral genome.

Materials and methods

Virus isolation

Virus isolation was performed from faecal samples during 1978-1985 from VAPP patients and healthy vaccinees in Hep-2 cells (Table 1) and the isolates were re-cultured for the purpose of the present study in Hep-2 cells. Initial isolation at that time occurred at the Hellenic Pasteur Institute and identification/serotyping was done by sero-neutralization with rabbit polyclonal antibodies, (National Institute for Public Health and Environment, RIVM, The Netherlands), according to enclosed instructions. To avoid viral mixtures serial ten-fold dilutions were performed; the last dilution presenting a complete cytopathic effect (CPE) was passaged once again in Hep-2 cells, and the serotype was retested as described above. Vaccine strains (Sabin types 1, 2, 3) used in this study were provided by the World Health Organization (WHO).

Genotyping and identification of recombinant OPV isolates

Primer pair UG52/UC53 was used for the identification of the vaccine origin and for the genotyping of the isolates in the 5'-NCR genomic region [28]. Moreover, the genotype of isolates in VP1 capsid protein was determined. The primer pairs used for complete VP1 gene sequencing were UG1/222 [29-30] for the first half of the VP1 gene and serotype-specific primer pairs $S_1$37/$S_1$688, $S_2$30/$S_2$688 and $S_3$26/$S_3$651 for Sabin 1, Sabin 2 and Sabin 3 isolates, respectively, for the second half of the VP1 gene [31].

The identification of recombination sites of isolates EP9, IF, ID, EP12, EP16, EP23 and the sequencing from 2C-3D genomic region have been previously published by our group [32-33].

Rct marker test

Reproductive capacities at different temperatures (Rct marker) were evaluated by Rct test. The Rct value is defined as the difference between the $\log_{10}$ virus titer of a viral stock measured at the optimal temperature (37^{o}C) and that of the supraoptimal temperature (40^{o}C). Briefly, each virus stock was decimally diluted through 10^{-7}. A 0.1-ml of medium containing 4 X 10^{4} Hep-2 cells was added into each well of two 96-well plastic plates. A 0.1-ml volume of each virus dilution was inoculated into four wells of each of the two plates. One plate was incubated at 37^{o}C and the other at 40^{o}C. After 5 days of incubation at the appropriate temperatures, the titers were determined by an endpoint micromethod [34] and were expressed in $\log_{10}$ 50% tissue culture infective dose units ($TCID_{50}$) per ml. Viruses were considered thermosensitive (ts) if the RCT value (between 37^{o}C and 40^{o}C) was greater or equal to 2.00 and thermo resistant (not-ts) when the RCT value was inferior to 2.00.

One-step growth curve experiment

One-step growth curve experiments were performed in Hep-2 cells at 37 and 40^{o}C, with a multiplicity of infection of 10 as determined by titration of virus stocks on Hep-2 cells, as has been previously described [35]. Briefly, 100 µl of Hep-2 cells (10^{4} cells/100 µl) were added into each well of twelve 96-well plastic plates. After the cells were attached to the plates, the isolates and the reference Sabin vaccine strains (Sabin 1, Sabin 2 and Sabin 3) were inoculated each into five replica wells of 96-well plastic plates. After the plates were rocked for 2 hours at 37^{o}C, the cells were thoroughly washed two times

each with 300 µl of Eagle MEM to remove unbound virus. Then, 100 µl of Eagle MEM was added into each well and the plates were incubated at 37^{o}C or 40^{o}C for 0, 1, 2, 4, 8 and 16 hours. The plates were subjected to three consecutive freeze-thaw cycles and the viral titers of the supernatants were determined by the $TCID_{50}$ assay on Hep-2 cells at 37^{o}C for each of the above mentioned hours post-infection. The titration was repeated five times for each of the post-infection hours.

Computational analysis

ClustalW was used for alignment of the sequences (nucleotide and amino acid) of 5'-NCR, VP1, 2C, 3A, 3B, 3C and 3D genomic regions of all virus isolates with those of the reference strains cited in GenBank (Sabin type 1: AY184219, Sabin type 2: AY184220 and Sabin type 3: AY184221). Amino acid sequences of VP1, 2C, 3A, 3B, 3C and 3D genomic regions of all virus isolates were obtained by using Gene-Runner V 3.05.

Results

Genotypes and recombination types of OPV isolates

The 5'-NCR and VP1 genotypes of isolates were identified. One strain was genotyped as Sabin 1, four strains as Sabin 2 and three strains as Sabin 3 (Table 1). Among the eight isolates, two (II and 729) were identified as non-recombinant and six (EP9, IF, ID, EP12, EP16 and EP23) were identified as recombinant in one site. The recombination type was S2/S1 for four isolates (EP9, IF, ID, EP12) and the recombination site was located in the 3D region for isolates IF and EP12, in the 3C region for isolate ID and in the 3A region for isolate EP9. Isolates EP16 and EP23 were S3/S2 and S3/S1 recombinant respectively and the recombination site was located in the 2C region (Table 1).

Temperature sensitivity of OPV isolates

The Rct marker assay is based on a comparison of the temperature-sensitive multiplication of poliovirus Sabin strains and the non-temperature-sensitive multiplication of virulent strains.

The non-recombinant Sabin-1 isolate II showed a partial reversion to non-ts phenotype with a Rct value of 2.0 units. Three of recombinant Sabin-2 isolates (EP9, IF, ID: S2/S1) showed a non-ts phenotype with Rct values ranging from 0.0 to 0.75 units while one (EP12: S2/S1) showed partial reversion to non-ts phenotype with Rct value of 1.75 units. Both recombinant Sabin-3 isolates, (EP16: S3/S2) and (EP23: S3/S1), showed partial reversion to non-ts phenotype with Rct values of 1.0 and 1.5 units, respectively. The non-recombinant Sabin-3 isolate 729 showed a ts phenotype with Rct value of 3.5 units (Table 2). The Sabin vaccine strains (Sabin1, Sabin2, Sabin3) exhibited, as expected, Rct values greater to 2.00 units.

One-step growth curve experiment

To determine whether the ts phenotype, as determined by Rct assay, was indicative of growth defects, one-step growth curve experiments were carried out for each isolate. We compared the growth rates and virus yields at 37^{o}C and 40^{o}C of each isolate with that of the corresponding genotype of Sabin vaccine strain (Sabin 1, Sabin 2 or Sabin 3) in one-step growth experiments in Hep-2 cells.

The non-recombinant Sabin-1 isolate II replicated with kinetics similar to that of its progenitor Sabin 1 vaccine strain showing that the viral yield rises after 2 hours post-infection at 37^{o}C (Fig. 1) while at 40^{o}C its growth was blocked as was also the case for Sabin 1 vaccine strain. However, the isolate II showed 1.0 $\log_{10}$ unit lower viral yield than Sabin 1 vaccine strain at

the initial stages of the infectious life cycle (0-2 hours post-infection) resulting in a difference of 2.5 $\log_{10}$ units at 4 hours post infection.

The recombinant Sabin-2 isolates EP9, EP12 and ID displayed similar growth kinetics to that of their progenitor Sabin 2 vaccine strain (Fig. 2). More specifically, they showed the same or lower viral yield than Sabin 2 vaccine strain during the infectious life cycle at both 37 and 40^{o}C. The recombinant Sabin-2 isolate IF also displayed similar growth kinetics to that of its progenitor Sabin 2 vaccine strain (Fig. 2). However, it showed higher viral yield than Sabin 2 vaccine strain at 4 and 8 h post-infection at 37^{o}C. Moreover, it showed 1.0-1.5 $\log_{10}$ units higher viral yield than Sabin 2 vaccine strain at 4, 8 and 16 h post-infection at 40^{o}C.

Both recombinant Sabin-3 isolates EP16 and EP23 showed different growth kinetics from that of their progenitor Sabin 3 vaccine strain (Fig. 3). Isolates EP16 and EP23 showed 2.0 and 1.7 $\log_{10}$ units, respectively, higher viral yield than the Sabin 3 vaccine strain at 0 and 1 hour post-infection. Moreover, the viral yield of isolates EP16 and EP23 rises after 1 hour post-infection at 37^{o}C while that of Sabin 3 vaccine rises after 2 hours post-infection. The isolates EP16 and EP23 replicated efficiently at both 37 and 40^{o}C. More specifically, the isolates EP16 and EP23 showed 2.0-3.5 and 1.0-2.0 $\log_{10}$ units, respectively, higher viral yield than Sabin 3 vaccine strain at both 37 and 40^{o}C during the infectious life cycle. The non-recombinant Sabin-3 isolate 729 replicated with kinetics similar to that of Sabin 3 vaccine strain at both 37 and 40^{o}C (Fig. 3).

Nucleotide and amino acid sequence analysis

In an attempt to correlate the phenotypic markers of the isolates with some molecular markers, we investigated the nucleotide and amino acid positions in

the genomes of the isolates that have been involved in the attenuated and thermo sensitive phenotype of Sabin vaccine strains.

The nucleotide sequences of 5'-NCR, VP1, 2C, 3A, 3B, 3C and 3D genomic regions were investigated in order to establish their respective predicted amino acid sequences. Tables 2 and 3 show the sequence differences with respect to Sabin vaccine strains. Mutations that result in loss of the attenuated and thermo sensitive phenotype of Sabin vaccine strains have been identified in the genomes of all isolates.

In non-recombinant Sabin-1 isolate II, the mutation U525C was identified (Table 2). The suppressive mutation U525C restores the stability of a base pair between nt 480 and 525 (AU in Mahoney, GU in Sabin 1, and GC in isolate II) in domain V of 5'-NCR and has been correlated with reversion to neurovirulence [6, 36]. The mutation C583G is situated in a double stranded region of domain VI of IRES and leads to a G-G mismatch. In VP1 coding region, the reversion at nt 2795 (A→G [Thr-106-Ala]), a well-known determinant of attenuation [6, 9] was also identified. Amino acid residue 106 is located at the exterior of the capsid, specifically in VP1 BC loop, and is involved in hydrophobic interactions with residue in position 90 [37]. Moreover, substitutions Ala-96-Thr and Lys-99-Asn were identified in isolate II. Both of them are highly exposed in the exterior of the capsid and are located in N-AgI site as well as in VP1 BC loop, which is participating in the formation of the north rim of the canyon and is involved in the binding of receptor CD155 to the virus [38-40]. Amino acid substitution Glu-168-Gly is exposed at the outer surface of the virion and is located in EF loop which is implicated in hydrophobic interactions with receptor CD155 [39-40]. Amino acid substitution Gln-220-Arg is situated in the exterior of the virion, in N-AgIIa site [38].

All Sabin-2 isolates displayed the mutation A481G in 5'-NCR (Table 2). This substitution is a reversion to the wild-type genotype and has been associated with increased neurovirulence and thermo resistance [8, 10, 41-45], probably by preventing a new base pair to form between residues 481 and 511, which would weaken the structure of 5'-NCR. Additionally, the mutation U398C was identified in two isolates (EP9 and IF) and the mutation U437C was identified in one isolate (EP9). These substitutions have also some effect in attenuation and thermo sensitivity of Sabin 2 vaccine strain even though minor [46]. Both of these mutations are situated in a double stranded region of domain IV of IRES and in particular they convert a base pair GU into GC. It is noteworthy the presence of mutation C584G in three Sabin-2 isolates (EP9, IF and EP12) which leads to a G-G mismatch. In VP1 coding region, amino acid substitutions in residue 143 were identified in two isolates (EP9 and EP12). Residue 143 is exposed on the external surface of the virion in the DE loop and the substitution of the attenuating Ile by Val, Thr or Asn has been correlated with increased neurovirulence [8, 10, 41, 42, 45, 47]. In isolate EP12, the substitution Phe-136-Cys was also identified. The residue 136 is situated in the hydrocarbon-binding pocket of VP1 interacting with a sphingosine molecule.

All Sabin-3 isolates displayed the mutation U472C in 5'-NCR (Table 2). This substitution is a reversion to the wild-type genotype and has been associated with increased neurovirulence and thermo resistance [7, 17, 48-50]. The nt 472 and 537 pair in the 5'-NCR in a predicted secondary structure. The attenuating U at nt 472 of 5'-NCR of Sabin 3 vaccine strain resulted in the alteration of a C-G base pair (nt 472-537) found in the neurovirulent wild strain P3/Leon/37 to a U-G base pair found in Sabin 3 vaccine strain, suggesting

that the attenuating phenotype is partially associated with a weakening of a base pairing in a highly conserved structure. It is noteworthy the presence of mutation C586G in all three Sabin-3 isolates. This substitution is situated in a double stranded region of domain VI of IRES and leads to a G-G mismatch as the mutations C583G in Sabin-1 isolate (II) and C584G in three Sabin-2 isolates (EP9, IF, EP12). These mutations may confer a selective advantage in OPV isolates as they have been also observed in isolates of previous studies [51-52]. In VP1 coding region, all three Sabin-3 isolates displayed the reversion at nt 2493 (C→U [Thr-6-Ile]) which is a determinant of attenuation [53-55]. Residue 6 is situated at the N-terminus of VP1 in the interior of the capsid and the substitution of the attenuating Thr by Ile probably strengthens the hydrophobic interactions between the VP1 amino terminus and endosomal cell membranes during virus cell entry [54, 56]. In isolate EP23, the substitution Ile-258-Val, partially exposed at the outer surface of the virion and near to N-AgI, was also identified.

In the 2C-3D non-structural genomic region of the isolates, nucleotide and amino acid substitutions were also identified (Table 3). The reversion at nt 6203 (C→U [His-73-Tyr]) of 3D polymerase coding region has been correlated with the attenuated and thermo sensitive phenotype of Sabin 1 vaccine strain [6, 10, 13, 25, 57, 58]. The C6203U mutation was identified in two recombinant Sabin-2 isolates (EP9: S2/S1 and ID: S2/S1) in a Sabin 1 background but was not identified in the recombinant isolate EP23 (S3/S1) which has also a Sabin 1 background in 3D region. The 2C coding region is known to contain a conserved stem-loop structure (nt 4443-4504 in Poliovirus type 2) serving as an essential cis-acting replicative element (CRE) involved in the uridylylation of VPg [59-60]. The substitution of either of the three A of a

conserved AAACA sequence (in Poliovirus type 1 and type 3) in the loop of the CRE reduces both VPg uridylylation and virus yield [60]. The substitution G4473A in isolate EP9 (S2/S1), converts the conserved AAGCA sequence of Sabin 2 vaccine strain to the conserved AAACA sequence of Sabin 1 vaccine strain. It has been proposed that the presence of G at nt 4473 is implicated in the attenuated phenotype of Sabin 2 vaccine strain [60].

Discussion

In this study, eight OPV isolates which were isolated from healthy vaccinees and VAPP cases during the time period 1978-1985, were investigated. More specifically, we determined the phenotypic traits such as thermo sensitivity and growth kinetics of each isolate and attempted to correlate them with genomic modifications.

The Sabin vaccine strains differ from their progenitors in biological properties other than neurovirulence, some of which have been used as in vitro markers to monitor the quality of new batches of vaccine prior to monkey neurovirulence assays. Temperature sensitivity and plaque size have been used as in vitro phenotypic markers in previous studies and were showed to correlate with the neurovirulence of poliovirus [6-10, 13, 25, 45, 58, 61]. MAPREC test which is based on the presence of mutations at known major determinants of attenuation has also been correlated with the neurovirulence of poliovirus [62]. One-step growth curve experiment was also used as an indication of viral multiplication ability that might correlate with viral virulence [9, 19, 63-65].

In the present study, the growth phenotype of eight OPV isolates (two non-recombinants and six recombinants) as well as of Sabin vaccine strains in Hep2 cells at two different temperatures (37^{o}C and 40^{o}C) was evaluated using

two different assays, RCT test and one-step growth curve analysis. In non-recombinant Sabin-1 isolate (II) the presence of mutations at known determinants of attenuation and thermo sensitivity or in other positions of 5'-NCR and VP1 regions involved in virus replication, does not favor the reversion of its growth phenotype at both 37^{o}C and 40^{o}C. It is noteworthy the lower virus titre (1 $\log_{10}$ unit) of isolate II than Sabin 1 vaccine strain at the first stages of the infectious life cycle (0 and 1 h post-infection). This initial difference leads to a lower virus titre of isolate II than Sabin 1 vaccine strain throughout the infectious life cycle and may originate from the different adsorption and/or penetration properties of isolate II in comparison to that of Sabin 1 vaccine strain. However, isolate II showed partial reversion to non-ts phenotype (Rct value=2.0). The presence of many other determinants of thermo sensitivity (nt 935, 1944, 2438 and 2741) in the 5' end of the genome of Sabin 1 vaccine strain makes difficult the reversion of Sabin-1 isolates to a non-ts phenotype [9].

All Sabin-2 isolates were S2/S1 recombinants. Isolates EP9, IF and ID showed a non-ts phenotype while isolate EP12 showed partial reversion to non-ts phenotype as it was proved by RCT test values. The different phenotypes of Sabin-2 isolates may correlate with the presence of mutations at nt 398 (isolates EP9 and IF) and at nt 6203 (isolates EP9 and ID). The presence of mutations at nt 481, 584 and 2909 in isolate EP12 seems to have minor contribution to the reversion to a non-ts phenotype. In the one-step growth curve test, all Sabin-2 isolates displayed similar growth kinetics to Sabin 2 vaccine strain. However, isolates EP9, ID and EP12 displayed lower virus titre (≤1 $\log_{10}$ unit) than Sabin 2 vaccine strain at the first stages of the infectious life cycle (0 and 1 h post-infection) as in the case of Sabin-1 isolate

II. Moreover, isolate IF showed higher viral yield than Sabin 2 vaccine strain at 4, 8 and 16 h post-infection at 40^{o}C. The discrepancy of the results of the two assays concerning the reversion of thermosensitivity may correlate with the fact that they show the viral multiplication for different time periods.

The recombinant Sabin-3 isolates EP16 (S3/S2) and EP23 (S3/S1) reverted partial to non-ts phenotype while the non-recombinant Sabin-3 isolate 729 displayed a ts phenotype as it seems from Rct test value. Isolates EP16 and EP23 replicated efficiently at both 37 and 40^{o}C in one-step growth curve test. More specifically, isolates EP16 and EP23 showed 2.0 and 1.7 $\log_{10}$ units, respectively, higher viral yield than the Sabin 3 vaccine strain at 0 and 1 hour post-infection. The presence of specific mutations (U472C, C586G in 5'-NCR and C2493U in VP1 coding region) and the recombination events may lead to different properties of isolates in comparison to Sabin 3 vaccine strain. These different properties may lead to the higher viral yield of isolates EP16 and EP23 at the initial stages of the infectious life cycle and to the earlier raise of virus titre than the Sabin 3 vaccine strain. The non-recombinant isolate 729 replicated with kinetics similar to that of Sabin 3 vaccine strain at both 37 and 40^{o}C. However, it showed higher viral yield (<1 $\log_{10}$ unit) than Sabin 3 vaccine strain at 0 and 1 h post-infection as isolates EP16 and EP23. The reversion at nt 2493 may be responsible for the higher viral yield of all three Sabin-3 isolates than the Sabin 3 vaccine strain at the initial stages of the infectious life cycle. Isolates EP16 and EP23 showed mutations at the same determinants of attenuation and thermosensitivity (nt 472 of 5'-NCR and 2493 of VP1) as the isolate 729. Consequently, the different phenotypes of isolates EP16 and EP23 in comparison with that of 729 are probably associated with the recombination event. This would be in

disagreement with a previous report by Macadam et al. 1989 [7] showing that similar type 3 recombinant viruses were slightly more ts than their non-recombinant counterparts containing the same reverted mutations.

Consequently, in non-recombinant Sabin-1 isolate the presence of mutations at known determinants of attenuation and thermo sensitivity does not affect its growth phenotype. Sabin 1 vaccine strain is genetically more stable than Sabin 2 and Sabin 3 vaccine strains and this fact may explain why Sabin 3 and Sabin 2 are more frequently isolated from VAPP cases than are Sabin 1 (only about 12% of cases) [66-67]. Moreover, the presence of S1/SX (SX: S2 or S3) recombinants is extremely rare. The sequences of Sabin type 1 origin are regularly found in SX/S1 recombinant strains [7, 45, 68-71]. The above may correlate with the increased fitness of SX/S1 recombinants than that of the S1/SX recombinants. The determinants of attenuation and thermo sensitivity of Sabin 1 vaccine strain are scattered along the whole genome but most of them are situated in the 5'-NCR and capsid region [9]. Sabin 1 vaccine strain may exchange the 5' end of its genome with that of Sabin 2 or Sabin 3 vaccine strains in order to get rid of these fitness-decreasing mutations.

In Sabin-2 isolates the presence of mutations at nt 398 of 5'-NCR and 6203 of 3D coding region favours considerably the reversion to non-ts phenotype and may lead to the reversion towards neurovirulence. The mutation at nt 398 of 5'-NCR has been frequently observed in Sabin-2 isolates from VAPP cases (8, 41, 72). The mutation at nt 6203 of 3D coding region has been also observed in Sabin-1 isolates from VAPP cases (14, 24, 45).

In Sabin-3 isolates, 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 [28, 32, 33, 68, 73-75].

Thus, both mutations and recombination could affect the phenotype traits of Sabin derivatives and may lead to neurovirulent viral strains. Moreover, the recombination allows either the congregation of reversed determinants of attenuation and thermo sensitivity in the viral genome or the elimination of some determinants of attenuation and thermo sensitivity from the viral genome.

In conclusion, both Rct assay and one-step growth curve analysis are effective markers for the estimation of neurovirulence of Sabin derivatives. Moreover the use of phenotypic markers such as the one-step growth curve analysis and the Rct assay along with the genomic analysis may shed additional light in the molecular determinants of the reversed neurovirulent phenotype of Sabin derivatives.

Acknowledgements

The work was supported by research grants of the Postgraduate Program “Applications of Molecular Biology-Genetics. Diagnostic Biomarkers”, code 3817, of the University of Thessaly, School of Health Sciences, Department of Biochemistry & Biotechnology.

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Table 1

Data for the OPV isolates.

Isolate	5'-NCR and VP1 genotype	Source	Recombination type	Crossover point	Accession numbers 5'NCR/VP1 /2C-3'NCR	Reference
II	S1	Cord tumor	nonrecombinant	-	EU598476/FJ609769/-	[32, 52]
EP9	S2	Healthy vaccinee	S2/S1	5314-5318/S2[a]	EU598464/EU598478 /AY736178	[33]
IF	S2	Healthy vaccinee	S2/S1	6247-6281/S2	FJ609757/FJ609772 /AY297764 (2C), AY297760 (3D)	[32]
ID	S2	VAPP	S2/S1	5521-5526/S2	FJ609758/FJ609773 /EU715814	[32]
EP12	S2	Healthy vaccinee	S2/S1	6337-6362/S2	EU598465/EU598479 /AY736179	[33]
EP16	S3	Healthy vaccinee	S3/S2	4892-4914/S3	EU598466/FJ609775 /AY736180	[33]
EP23	S3	VAPP	S3/S1	4880-4887/S3	EU598468/EU598480 /AY736181	[33]
729	S3	Healthy vaccinee	nonrecombinant	-	EU598477/FJ609778/-	[52]

[a]Numbering according to Sabin 2 (AY184220) or Sabin 3 (AY184221) vaccine strains.

Table 2

Location of mutations in 5'-NCR and VP1 sequences of OPV isolates and comparison with the corresponding nucleotide and amino acid positions in Sabin vaccine strains. Numbering according to vaccine strains Sabin 1(AY184219), Sabin 2 (AY184220) and Sabin 3 (AY184221). Mutations identified in more than one isolates are underlined. Reproductive capacity values of OPV isolates and Sabin vaccine strains at different temperatures (Rct marker) are also indicated.

Isolate	Recombination type	Mutations		Δ logRct 40
		5'-NCR	VP1	
II	S1 nonrecombinant	525 U-C 583 C-G	2605 G-A - 2765 G-A 96-Ala-Thr 2776 G-U 99-Lys-Asn 2795 A-G 106-Thr-Ala 2917 C-U - 2982 A-G 168-Glu-Gly 3138 A-G 220-Gln-Arg	2.0
EP9	S2/S1	398 U-C 437 U-C 481 A-G 584 C-G 576 C-U	2909 U-A 143-Ile-Asn 3252 C-U -	0.75
IF	S2/S1	398 U-C 481 A-G 584 C-G	-	0.0
ID	S2/S1	481 A-G 567 U-A	-	0.0
EP12	S2/S1	481 A-G 597 U-A 584 C-G	2859 A-G - 2888 U-G 136-Phe-Cys 2909 U-C 143-Ile-Thr	1.75
EP16	S3/S2	472 U-C 586 C-G	2493 C-U 6-Thr-Ile 2608 A-G - 3259 A-G -	1.0
EP23	S3/S1	472 U-C 586 C-G	2493 C-U 6-Thr-Ile 3248 A-G 258-Ile-Val 3352 A-G -	1.5
729	S3 nonrecombinant	248 G-A 472 U-C 586 C-G	2493 C-U 6-Thr-Ile 2659 G-A - 2716 G-A -	3.5
Sabin 1	-			3.5
Sabin 2	-			3.0
Sabin 3	-			3.5

Table 3

Location of mutations in 2C-3D genomic region of isolates EP9, IF, ID, EP12, EP16, EP23 and comparison with the corresponding nucleotide and amino acid positions in Sabin vaccine strains. The genomic region from 2C-3D of no recombinant isolates II and 729 was not sequenced. Numbering according to vaccine strains Sabin 1 (AY184219), Sabin 2 (AY184220) and Sabin 3 (AY184221). Mutations identified in more than one isolates are underlined.

Isolate	Region	Origin	Mutations
EP9	**2C**	S2	4473 G-A -
IF		S2	4842 G-A -
ID		S2	4456 G-A 112-Val-Ile
EP12		S2	4773 C-U -
			4174 C-G 18-Leu-Val
			4992 U-A -
EP16		S3/S2	-
EP23		S3/S1	-
EP9	**3A**	S2/S1	5130 U-C[a] -
IF		S2	-
ID		S2	-
EP12		S2	-
EP16		S2	-
EP23		S1	5191 A-G -
EP9	**3B**	S1	5389 A-U -
IF		S2	-
ID		S2	-
EP12		S2	-
EP16		S2	-
EP23		S1	-
EP9	**3C**	S1	5665 U-C -
			5698 A-C 87-Arg-Ser
			5720 A-C 95-Thr-Pro
			5760 A-G 108-Lys-Arg
			5959 G-A -
IF		S2	-
ID		S2/S1	-
EP12		S2	-
EP16		S2	-
EP23		S1	5791 U-C -
EP9	**3D**	S1	<u>6203</u> C-U 73-His-Tyr
			6217 A-G -
IF		S2/S1	-
ID		S1	<u>6203</u> C-U 73-His-Tyr
			6373 U-C -
EP12		S2/S1	6781 C-U[a] -
EP16		S2	6219 G-A -
			6327 U-C -
EP23		S1	6474 G-A 163-Arg-Lys
			6484 A-U -

[a]Numbering according to Sabin 2 (AY184220) or Sabin 1 (AY184219) vaccine strains.

Fig.1. One-step growth curve analysis of Sabin-1 isolate II in comparison with Sabin 1 vaccine strain in Hep2 cells. Cells were infected at an MOI of 10 and incubated at 37^{o}C or 40^{o}C. Total virus production at different times (0-16 hours) post-infection was determined by $TCID_{50}$ assay on Hep2 cells. Each point represents the mean (±standard deviation) of virus titers from five different experiments.

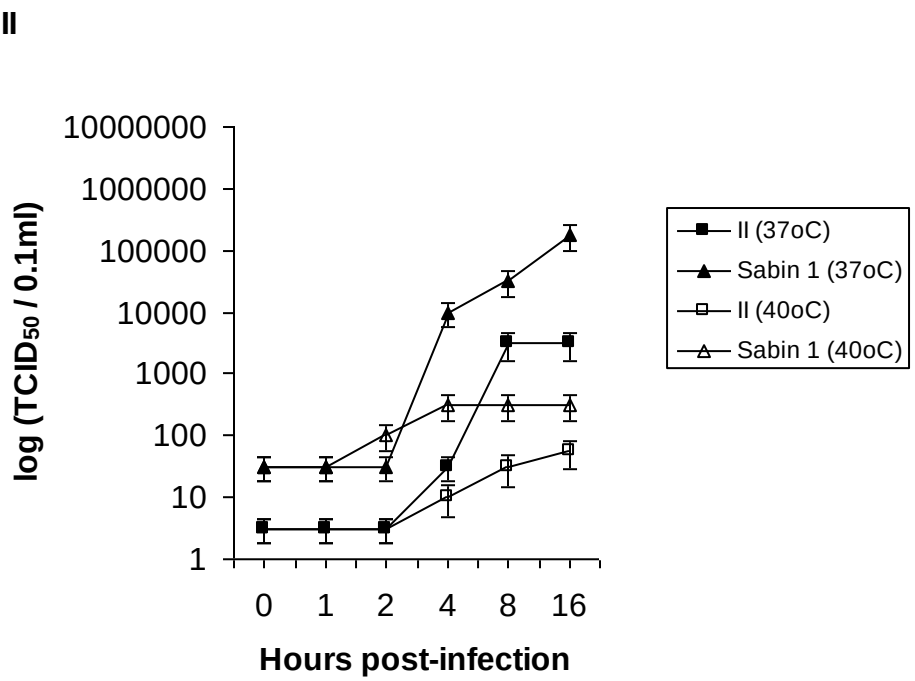
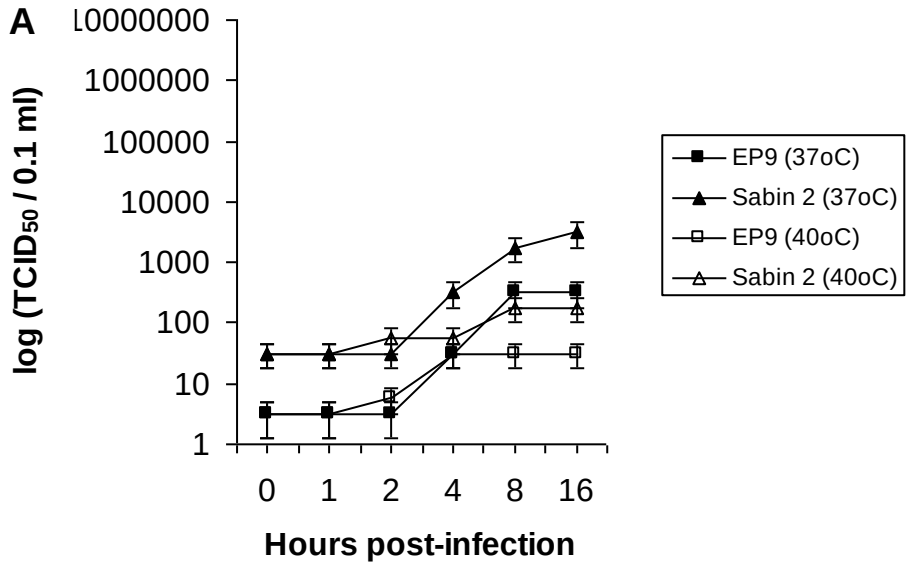
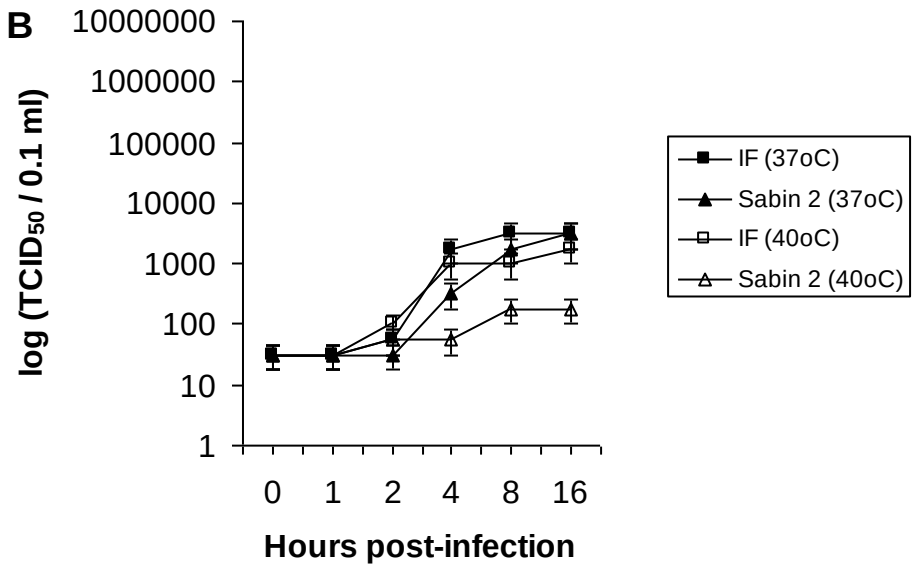


Fig.2. One-step growth curve analysis of Sabin-2 isolates EP9 (A), IF (B), ID (C) and EP12 (D) in comparison with Sabin 2 vaccine strain in Hep2 cells. Cells were infected at an MOI of 10 and incubated at 37^{o}C or 40^{o}C. Total virus production at different times (0-16 hours) post-infection was determined by $TCID_{50}$ assay on Hep2 cells. Each point represents the mean (±standard deviation) of virus titers from five different experiments.

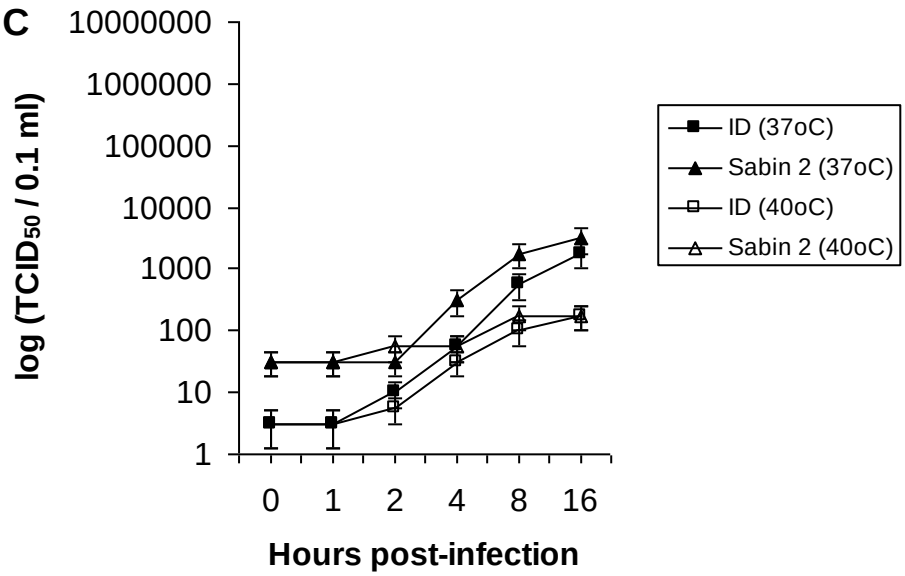
EP9



IF



ID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1
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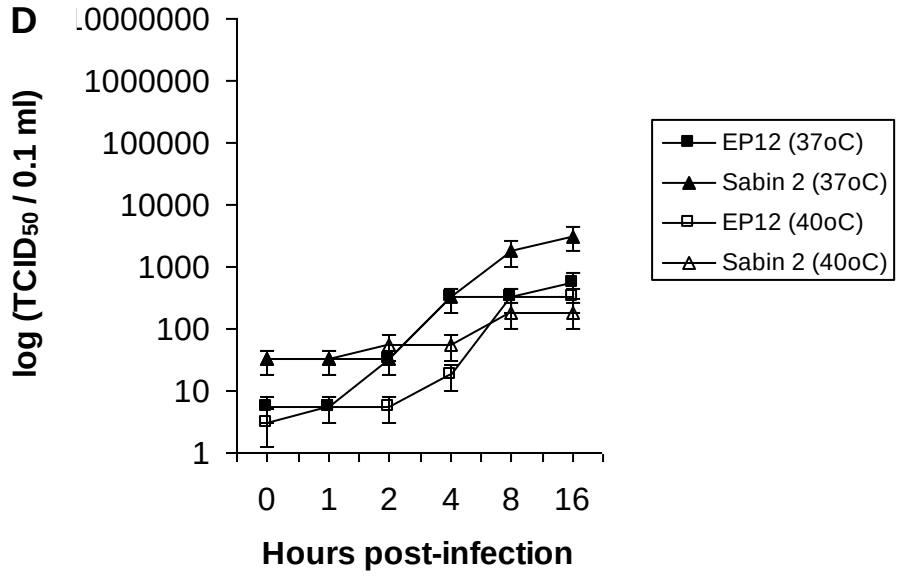
Hours post-infection
ID (37oC)
Sabin 2 (37oC)
ID (40oC)
Sabin 2 (40oC)

EP12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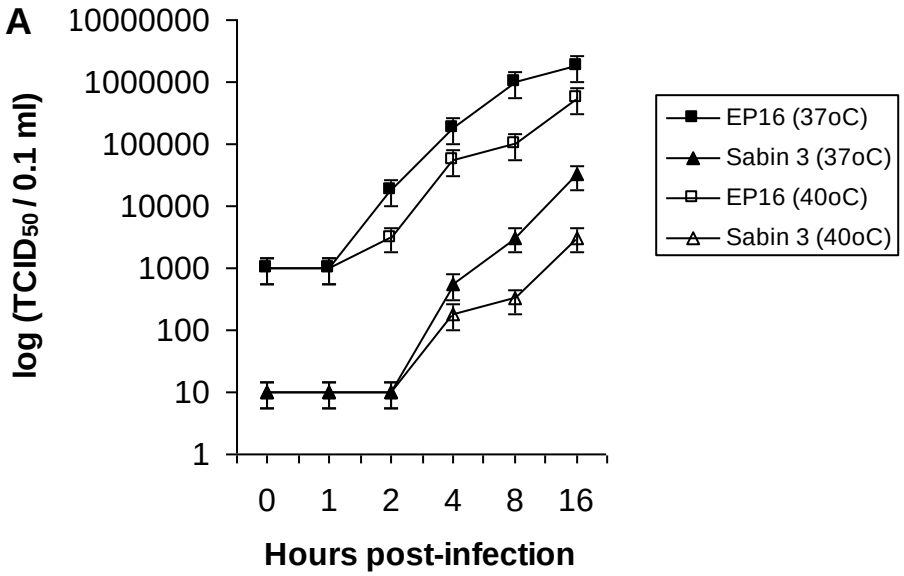
8
16
Hours post-infection
EP12 (37oC)
Sabin 2 (37oC)
EP12 (40oC)
Sabin 2 (40oC)

Fig.3. One-step growth curve analysis of Sabin-3 isolates EP16 (A), EP23 (B) and 729 (C) in comparison with Sabin 3 vaccine strain in Hep2 cells. Cells were infected at an MOI of 10 and incubated at 37^{o}C or 40^{o}C. Total virus production at different times (0-16 hours) post-infection was determined by $TCID_{50}$ assay on Hep2 cells. Each point represents the mean (±standard deviation) of virus titers from five different experiments.

EP16



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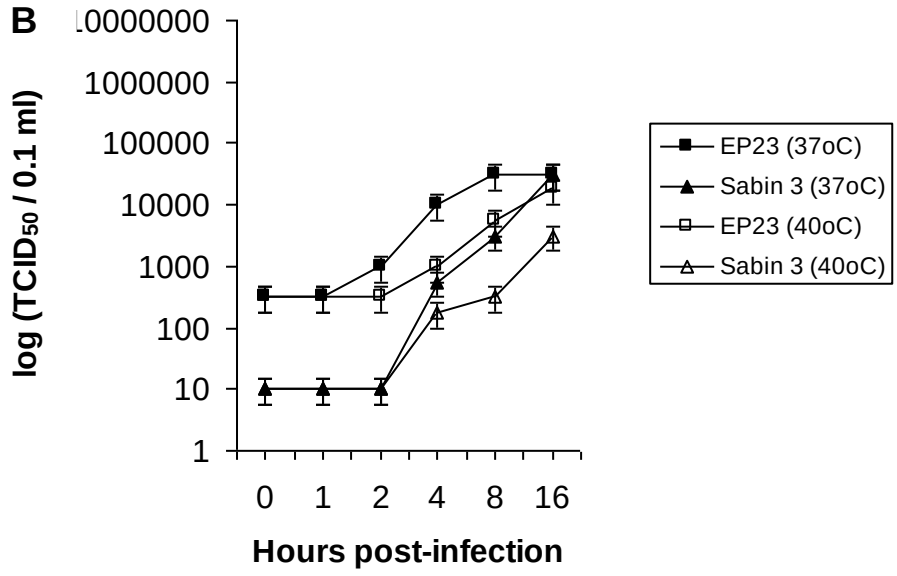
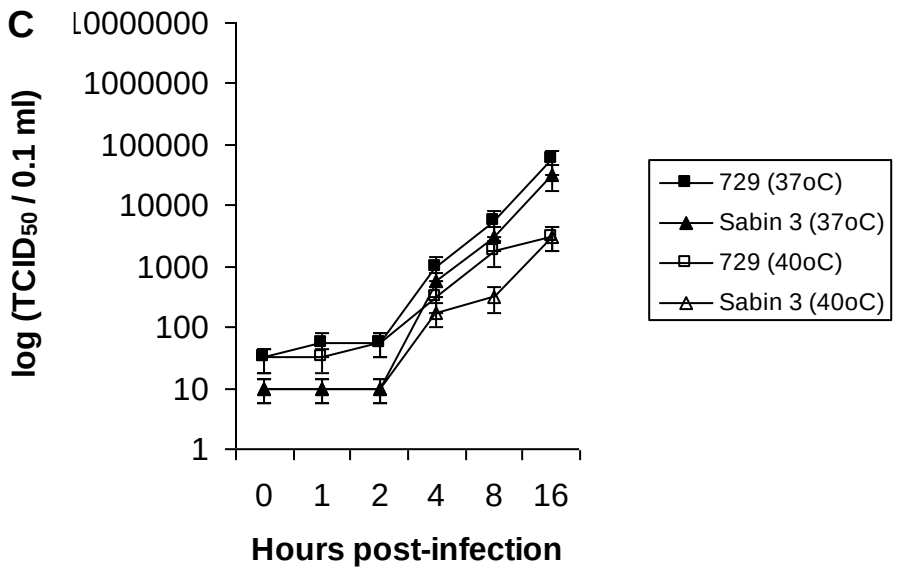


729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4
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16
Hours post-infection
729 (37oC)
Sabin 3 (37oC)
729 (40oC)
Sabin 3 (40oC)