Iranian Journal of Virology 2008; 2 (1&2)

GENOMIC ANALYSIS OF VACCINE – LIKE POLIOVIRUS ISOLATES FROM STOOL SPECIMENS OF ACUTE FLACCID PARALYSIS CASES WITH RESIDUAL PARALYSIS DURING THE YEARS 1380-1381 IN IRAN

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Abstract: The live attenuated oral poliovirus vaccine(OPV),used for many years has reduced the poliomyelitis cases throughout the world wide. Most RNA viruses have highly mutable genomes and polioviruses are among the most rapidly evolving of all RNA viruses. Mutations naturally occurs during their replication in human intestine and in rare instances it can be back mutation to become to neorovirulant(vaccine-associated paralytic poliomyelitis). For many years the role of some critical nucleotides in 5`non-coding region in vaccine-associated paralytic poliomyelitis was known however the investigators have focused on the importance of other nucleotides in capsid regions such as VP1.Therefore ,mutations in this region of polioviruses isolated from acute-flaccid paralysis cases were analyzed. In Iran the WHO eradication program has been done since 1373 and all acute flaccid paralysis have been studied .During the years 1380-1381 out of all AFP cases, 5 patients were found to have residual paralysis and 5 vaccine-like polioviruses were isolated from these patients .Using icroneutralization test and intratypic differentiation tests (ELISA, probe-hybridization and RT-PCR).Three isolates (no:2,10 and11) were vaccine-like polioviruses type2. The other isolates (no:7 and 9) were vaccine-like polioviruses type3. The VP1 regions of these isolates were sequenced and analyzed.Sequence analysis showed that 3 vaccine-like polioviruses type2(no:2,10,11) had 1and 2 nucleotide substitutions in VP1 region in comparison with reference Sabin strain type2 respectively and in vaccine-like polioviruses type3(no:7,9) there was only one nucleotide substitution in isolate7 in this region in comparison with reference Sabin strain type 2 . Eradication of wild polioviruses is near however it is essential to identify any mutations in all three Sabin strains and improve our knowledge about molecular pathogenesis of these viruses.

Keywords: • Vaccine • like poliovirus, residual paralysis, VAPP: Vaccine • associated paralytic poliomyelitis, AFP: Acute • flaccid paralysis.

Introduction

Polioviruses 1, 2 and 3 the three serotypes of the Enterovirus genus from the Picornaviridae family are the main causative agents of poliomyelitis.

These viruses consist of icosahedric particles composed of 60 copies of each of four capsid proteins, VP1, VP2, VP3 and VP4 surrounding the viral genome, which is a single stranded RNA of positive polarity of about 7500 nucleotides (Wimmer et al,1993).

The RNA molecule contains a 5′- non coding region (5′- NCR) of about 740 nucleotides, a single open reading frame (ORF) coding for structural and non – structural proteins, and a 3′ non – coding region (3′- NCR) of about 70nucleotides followed by a poly (A) – tract. The error frequency of thisgenomic RNA during the replication by RNA polymerase is high (Balanant et al, 1991). Because of this inherent

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[DOI: 10.21859/isv.2.1.2.1]

property the live attenuated oral poliovirus vaccine used for more than four decades to interrupt poliovirus transmission and the vaccine of choice for developing countries is generally unstable (Friedrich et al, 2000 and Kew et al, 1981) with disadvantages rarely causing poliomyelitis (Friedrich et al ,2000 and Kew et al,1981,2005). Vaccine – associated paralytic poliomyelitis (VAPP) is clinically undistinguishable from poliomyelitis caused by wild polioviruses. VAPP cases are sporadic and occur in both OPV recipients and their unimmunized contacts. In fact, VAPP is a direct clinical consequence of the genetic instability of the Sabin OPV strains.

The world Health Organization (WHO) has focused on the surveillance and specimen testing of acute flaccid paralysis (AFP) cases in children less than 15 years (Alexander et al,1997. Aylward et al,2004.Balanant et al,1991.Center for Disease control and prevention,2000).

In Iran mass vaccinations in National Immunization Days (NID) and the

AFP clinical surveillance programs have been successfully established in according to the WHO program the immunization coverage was 100% in 2002 (MMWR; DEC 14, 2001). Surveillance for polioviruses has two arms:

(i)Detection and clinical investigations of AFP cases.

(ii)Virologic studies of polioviruses isolated from their samples. Each year about 300- 400 AFP cases were identified and less than 1/3 of them had residual paralysis and 5 Sabin-like polioviruses were isolated from them. In this study we describe the genomic analysis accomplished on these isolates.

Materials and Methods

Patients

All patients were AFP cases with residual paralysis. 5 Sabin strains isolated from them and definite diagnosis of residual paralysis was established according to WHO recommendation. All patients were immunocompetent and had received three doses of oral polio vaccine. Table 1 summarized the specifications of patients.

Lab No.	Age (m)	province	Sex	Last Vaccination date	Onset date	Specimen date	*RT-PCR	●ELISA
2	40	Kermanshah	M	16 Apr 01	04 Jun 01	19 Jun 01	PV2-S	PV2-SL
7	36	Khorasan	F	11 Nov01	18 Nov 01	22 Nov 01	PV3-S	PV3-SL
9	43	Boushehr	M	28 Jan 02	07 Feb 02	18 Feb 02	PV3-S	PV3-SL
10	3	Tehran	F	02 May 02	18 May 02	29 May 02	PV2-S	PV2-SL
11	15	Sistan and Baluchestan	F	10 Nov 02	19 Nov 02	28 Nov 02	PV2-S	PV2-SL

Table 1. Specifications of five acute flaccid paralysis cases with residual paralysis during the years 2001 – 2002 and the results of intratypic differentiation tests of the polioviruses isolated fro them

***S:** Sabin strain poliovirus.

●SL: Sabin-Like poliovirus.

Specimens

two stool samples were taken within 48h of each other not later than 14 days after the onset of paralysis. The patient's specimens were transported to our laboratory in cool conditions in cold boxes(-10 to -5°c) and stored at - 20°c before inoculation on tissue cultures.

Virus isolation and serotyping

fecal specimens were processed for virus isolation and identification on RD (human embryo rhabdomyosarcoma) and $L_{20}B$ (mouse L cells expressing the human poliovirus receptor) and Hep_2 – CinCinnati (epidermoid carcinoma human Larynx) cell lines – recommended by WHO. Specimens were considered positive if cytopathic effect was detected after 2 passages during 14 days of inoculation, then isolated viruses were typed by seroneutralization of cytopathic effect using pools of antisera against polioviruses and other enteroviruses as recommended by WHO (mannual,2004).

Intratypic differentiation (ITD)

In order to establish whether the poliovirus isolates were of vaccine or wild origin, they were tested by three methods recommended by the WHO for polio surveillance: (I) Enzyme – linked immunosorbant assay proposed by WHO for polio surveillance: allowing detection of antigenic differences between non – Sabin and Sabin – derived strains, (ii) a hybridization assay using riboprobs hybridizing specifically with the genome

[DOI: 10.21859/isv.2.1.2.1]

of vaccine – related isolates and(iii) a reverse transcription – PCR amplifying test was performed to identify each serotypes (World Health Organization ,2004).The RT – PCR products were loaded on 1.5% gel Agaros. All isolates were stored at - 70°C in microcentrifuge tubes containing 1 – 2.5ml suspension of $L_{20}B$ cell culture.
These isolates were sent to Center for Disease Control and prevention (CDC) to be sequenced.

Viruses
Sabin strains of types 2 and 3 with GenBank accession numbers Ay082679 and Ay082683 were used as reference sequences respectively.

Sequence Analysis
Nucleotide sequence data from each isolates was analyzed using Clustal W program.

Results

Using micronutralization test, 3 isolates (2, 10 and 11) were found to be poliovirus type 2 and the two others (7 and 9) were identified as poliovirus type 3. In all three intratypic differentiation tests (ELISA, probe- hybridization and RT–PCR) all isolates were identified as Sabin strains. Table 1 summarizes the results from primary tests. Isolate 2 had 99.9% identity and isolates 10 and 11 had 99.8% identity in VP_1 region with Sabin strain 2 (Ay082679). Isolate 9 had 100% identity in VP_1 region with Sabin strain 3 (AY 082683) and isolate 7 had 99.9% identify with this Sabin strain in VP1 region. There was only one nucleotide substitution in VP_1 region (A2908G) in isolate 2. In isolates 10 and 11, two nucleotide substitutions were found: T2909 A, C 3231T and T 2909C, T3339C respectively.
Isolate 7 had one nucleotide substitution C 2493 T. The results of alignment and characteristics of nucleotide substitutions in VP_1 region of each isolate were summarized in Tables 2 and 3.

Virus No. / NT Position	*AY082679	2	10	11
2908	A	G	A	A
2909	T	T	A	C
3231	C	C	T	C
3339	T	T	T	C

Table 2. Position and the type of nucleotide substitutions in VP1 region of each isolates in comparison with reference Sabin strain type 2.

* GenBank accession number of reference Sabin strain.

Virus No. / Nucleotide Position	*AY082683	7	**9
2493	C	T	C

Table 3. Position and the type of nucleotide substitution in VP1 region of isolates 7 and 9 in comparison with reference Sabin strain type 3.

* GenBank accession number of Sabin strain type 3.
** Isolate 9 had %100 identity in VP1 region with Sabin strain type 3.

Comparison between amino acid residues in VP_1 region of each isolate and reference viruses resulted in finding amino acid changes in this region. Isolates 2, 10 and 11 had different amino acid residues in position 143 of VP_1 protein. Table 4 shows the substitutions in amino acid residues of isolates 2, 10 and 11 in comparison with reference virus (AY082679).
Isolate 7 had an amino acid substitution I → T (AY082663 → 107) in sixth residue of VP_1 protein.

Virus No.	**AMINO ACID**
*AY082679	Isoleucine
2	Valine
10	Threonine
11	Aspargine

Table 4. Amino acid substitution in position 143 of VP1 protein in isolates2, 10 and 11 in comparison with reference Sabin strain type 2.
*Genebank accession number of Sabin strain type 2.

Discussion

Mutations occur within the viral genome of the Sabin polioviruses as part of the natural replication process (Balanant et al,1991. Friedrich,2000). In rare circumstances these mutations, may result in reversion to neurovirulence (De,L,B.K.Nottay et al,1995.Kew et al ,1981 and 2005). Acute-flaccid paralysis can caused by Sabin – like polioviruses which differ from the parental Sabin strains by less than 1% inVP_1 region, Vaccine-derived polioviruses which differ from the parental Sabin strains at 1% to 15% of VP_1 nucleotides and by wild polioviruses which differ from the Sabin strains at >15% in VP_1

[DOI: 10.21859/isv.2.1.2.1]

region (Fine,P.E and I.A.M. Carneiro, 1999. Friedrich, 2000. Kew et al,2005).

Vaccine-derived polioviruses are viruses with unusual genetic properties, demonstrate higher genetic divergence from OPV strains than do isolates from most vaccine–associated paralytic poliomyelitis (VAPP) cases (Kew et al, 2005). Presumably vaccine – related isolates with low genetic diversity by <0.5% from the parental vaccine strain have short duration of infection in immunocompetent individuals (Festus Doyin Adu et al,2003.Javier Martin et al,2002.M.Equestre et al,1991). In our country, during the years 2001 – 2002 there were a few AFP cases with residual paralysis using ELISA, probe- hybridization and RT – PCR tests 5 Sabin strains were isolated from them. Genomic analysis revealed less than 1% divergence in VP_1 region in comparison between our isolates and reference Sabin strains (Tables 1 and2). So the patients were identified as vaccine – associated paralytic poliomyelitis. Isolates 2, 10 and 11 had a different amino acid residue at position 143 in VP_1 region. Diversity in this position can cause reversion to neurovirulence(Festus Doyin Adu et al,2003.J.M.Tatem et al,1992. Stuart R.Pollard et al, 1989). In Sabin strain type 2 according to the studies in monkeys and mice, inoculation of these revertant Sabin strain type 2 resulted in paralysis (Kew et al, 1981). Other studies of determinants for attenuation and reversion in Sabin strain type 2, proposed that changing in amino acid 143 in VP_1 region, constitutes the sole variation in otherwise perfectly conserved domain and results in a conformational change of the viral surface and increase its interaction with neural cells but not epithelial cells (Festus Doyin Adu et al, 2003, J.M.Tatem et al, 1992, Moss,E.G et al,1989. M.Equestre et al, 1991. Stuart R.Pollard et al,1989).With two other isolates 7 and 9, there was only one substitution (C 2493T) in VP_1 region which resulted in an amino acid substitution in position 6 in VP_1 protein (I→T).This amino acid substitution has been proved as a determinant of neurovirolence in monkeys and mice (Mark A.Pallansch et al,2000).

It is also proposed that, this change can result in concomitant decrease in the hydrophobicity of the protein is buried inside the virions and forming a network with terminal regions of the other capsid proteins, it has an important role in stabilizing the virion assembly (Harber,J et al,1995. J.M.Tatem et al, 1992.). With another isolate (9) there was no mutation in VP_1 region but because of the clinical manifestations (AFP with residual paralysis), existence of mutations in other parts of the genome can not be precluded so this isolate needs to be more studied (Mark A.Pallansch et al, 2000).We compared our isolates with other isolates from epidemics of vaccine-derived polioviruses type2 in Egypt ,Nigeria and Mongolia. Although those isolates had more than 1% nucleotide differences in the VP1 region from Sabin strain type 2 however, we could find the same mutations in position143 in VP1 protein .With poliovirus type3 there was no epidemic report of vaccine-derived polioviruses type 3(vdpv3) and there were only a few type3 polioviruses isolated from immunodeficient patients or environment and all of them had an amino acid substitution in position 6 in the VP1 protein. Currently, there is urgent debate about the best method of stopping vaccination against poliomyelitis once the wild – type poliovirus has been eliminated completely and the vaccine – strain viruses are going to be the only sores of live polioviruses in the world.

Acknowledgments

We thank Dr. Olen Kew, Dr. David Kilpatrick and their colleagues for sequencing of the isolates. This project was partly supported by Polio National Laboratory, Division of Virology, Department of Pathobiology, School of public Health, Tehran University of Medical Sciences.

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[DOI: 10.21859/isv.2.1.2.1]