VNU Journal of Science, Natural Sciences and Technology 23 (2007) 145-151

Using improved Non Ri -Maprec assay to detect virulence mutations in poliomyelitis Vaccine viruses: advantages over *rct*40 test

Thieu Thi Hoai Anh*, Le Thi Luan

Poliomyelitis vaccine Research and Production center (Poliovac), 135 Lo Duc, Hanoi, Vietnam

Received 23 January 2006

Abstract. Mutant analysis by polymerase chain reaction and restriction enzyme cleavage (MAPREC) has been developed by Chumakov *et al.* for poliovirus to determine quantitatively the presence of genomic changes in particular nucleotide sequences that lead to virulence phenotypes of oral poliomyelitis vaccine (OPV or live attenuated vaccine) [1]. Afterwards, this RI (radioisotope) MAPREC was developed to non RI – MAPREC by Japan Poliomyelitis Research Institute in 1998 to compensate for some disadvantages of RI-MAPREC [2]. This test was highly recommended for laboratories to assess the safety of their vaccine products. At Poliovac, the *rct*40 test was used to be considered a major tool for vaccine products examination. In this research, we have improved the non RI MAPREC test to apply it well with practical conditions in our laboratory and compared this test to the usual *rct*40 test to point out its great advantages.

1. Introduction

Poliomyelitis is an acute disease of the central nervous system (CNS) caused by poliovirus. Oral poliomyelitis vaccine (OPV) is usually considered the major tool for eradication of poliomyelitis in many countries, as well as Vietnam. It is regarded as one of the safest vaccines in current use, but some polio vaccine related - paralytic cases have been reported recently [3]. These cases are caused by vaccine viruses undergoing reverted mutations during repeated replications.

Poliovirus has a genome of single-stranded RNA, its rate of mutations is usually high during replication since RNA-dependent RNA polymerase does not have the proof – reading activity. Thus strict control is strongly required during vaccine production.

Although there are many alterations between virulent and normal forms of polioviruses: for Sabin type 1, 2 and 3, vaccine strains differ from their progenitors by 55, 23 and 11 mutations, respectively [4], few changes required for attenuation and reversion. That is the reason why type 3 Sabin strain has the least stable genome and thus can easily revert to virulent form while Sabin type 1, in contrast, has the most stable genome. It was demonstrated that neurovirulence increased when the following changes took place in base positions of the viral genome: in serotype 1, position 480 in the non coding region (NCR) changed from G to A, position 525 changed from U to C and therefore compensated for the presence of G-480 by restoring base-pairing in

* Corresponding author. Tel.: 84-4-8531213.
E-mail: tthanh@vnmilk.com.vn

the stem-loop structure [5]; for serotype 2, position 481 changed from A to G, prohibiting the formation of a new base pair between residues 481 and 511 that weakens the secondary structure [5]; for type 3, position 472 changed from U to C, restoring the polypyrimidine tract-binding protein (an initiation factor in neuroblastoma cells [6]) site. All these nucleotides lay within the region designated "internal ribosomal entry site" (IRES), of which the stability has a considerable influence on the translation efficiency of virus-specific proteins associated with neurovirulence, in other words, these mutations interfere with the IRES ability to interact with *trans*-acting factors.

Chumakov *et al.* introduced a method designated "Mutant analysis by polymerase chain reaction and restriction enzyme cleavage" (MAPREC) to estimate the ratio of viruses containing genes of a virulent nature in a vaccine virus population. When the proportions of such genes exceed a particular level, the vaccine fails the monkey neurovirulence test (MNVT): for type 1, approximately 5% of 480-A and 525-C combined; for type 2, between 1.7% and 3.7% of 481-G; for type 3, about 1% of 472-C. At present, MAPREC can be used as a supplement to the MNVT for several purposes: establishing and monitoring of molecular consistency of vaccine production, as a preliminary molecular test before MNVT, as a substitute of the *rct*40 test, screening of single harvests and monitoring the genetic stability of vaccine viruses.

The non-RI MAPREC test was developed from RI-MAPREC by Japan Poliomyelitis Research Institute in 1998 to compensate for the biggest disadvantage of RI-MAPREC: its use of radioisotope requires a high level of expertise and the use of equipment, which, unlike in economically developed countries, may not be available in developing countries which are threatened the most by poliomyelitis. A further consideration is that its use carries the risk of causing environmental problems [2].

The *rct*40 test (reproductive capacity at 40°C) is used to be considered a simple tool for assessing the virulence of vaccine viruses via their temperature sensitivity. The revertant is not temperature - sensitive, therefore its reproductive capacity has no difference at the temperatures of 36°C and 40°C. Thus, by examining the reproductive capacity of vaccine viruses at these temperatures and comparing the results, the genetic material of the vaccine can be indirectly assessed. However, mutations that have the greatest contribution to virulence have only minor effect on viral temperature sensitivity but other nucleotides (6203, 7071, 7410 and 7441 for type 1; 2034 for type 3) [4,5,7]. Therefore, if a single harvest shows temperature sensitivity, it does not mean totally this sample is not neurovirulent. This great disadvantage of *rct*40 test can be definitely overcome by MAPREC.

In this study, we performed improved non RI-MAPREC assay on type 3 vaccine viruses, which have the greatest possibility of revertant mutation to assess their safety and compared this test to the usual *rct*40 test in terms of effectiveness, exactitude, time consumed and expenses. On the basis of these results, we recommended one of these two tests for the *in vitro* examination of oral polio vaccine's safety in our laboratory.

2. Materials and methods

2.1. Viral RNA extraction and cDNA synthesis

In this study we used six type 3 polio virus single harvests produced in 2005 at Poliovac designated 402, 403, 404, 405, 406, 407 and 4

marker samples designated 472-a, 472-b, 472-c, 472-d provided by Japan Poliomyelitis Research Institute.

Positive single-stranded RNA of poliovirus was extracted from 0.5 ml virus suspension by phenol extraction with sodium dodecyl sulfate (SDS) of final concentration of 0.5%. The viral RNA was precipitated using 100% isopropanol and treated with ethanol 70%, cDNA was then synthesized with Moloney murine leukemia virus reverse transcriptase (MMLV-RT – Invitrogen), random hexanucleotide primers (Fermentas) and incubated for 1h at 37°C.

2.2. MAPREC, non RI-MAPREC and improved non RI-MAPREC

Both MAPREC and non RI-MAPREC are quite identical except for the way these two methods using to quantify digested DNA bands. For poliovirus type 3, a virulent base C at residue 472 was examined in our research. Since no known restriction enzyme cuts either attenuated or reverted poliovirus sequences at nucleotide 472, a restriction site of *Mbo*I is engineered by PCR using a mutagenic primer. Thus the restriction site is composed, in part, of the primer sequence and in part, of the viral cDNA used as a template.

Table 1. PCR primers used to detect mutations by MAPREC and non RI-MAPREC

*MAPREC

Primer polarity	Primer sequence
sense	T431GAGCTACATGAGAGTgCTCCGGCCCCTGAATGCGGCTgA470
antisense	C513AGGCTGGCTGCTGGGTTGCAGCTGCCTGC484

Lowercase letters show modifications compared to the poliovirus sequence which were introduced to create or destroy restriction sites. The normal vaccine sequence was cut by *Hin*fI, the revertant sequence was cut by *Mbo*I. A g at position 447 was introduced to destroy restriction site for *Hin*fI site while a g at position 469 was inserted to create restriction sites for both *Hin*fI and *Mbo*I.

*nonRI-MAPREC

Primer polarity	Primer sequence
sense	T440GAGAGTCCTCCGGCCCCTGAATGCGGCTgAT471
antisense	A532CGGACTTGCGCGTTACGACAGGCTGGCTGC502

Unlike MAPREC, in non RI-MAPREC, only *Mbo*I was used to cut the revertant sequence. PCR amplification using sense and antisense primers as described previously and *Taq* DNA polymerase (Invitrogen, Perkin-Elmer) was conducted for 40 cycles.

For RI-MAPREC, PCR amplification was asymmetric (i.e. a 10-fold excess of sense primer ensures that the predominant product of this reaction is single-stranded DNA: 30µg/ml for sense primer and 3µg/ml for antisense primer). The second strand is synthesized using a ^{32}P labeled antisense primer. After treatment of the amplified DNA product with *Mbo*I, the digested material was separated by electrophoresis in polyacrylamide gel. The mutational change (percentage of 472C) was calculated by measuring radioactivity in counts per minute (cpm) of digested and undigested bands, using the equation: digested DNA band (cpm) over digested DNA band (cpm) plus undigested DNA band (cpm).

For non RI-MAPREC, PCR amplification was performed using the primers shown in Table 1, with sense and antisense primers at equimolarity (both at a final concentration of 3μg/ml) to have the PCR product of 93bp. After treatment of 25μl of the DNA product with the composition of *Mbo*I and its buffer (Fermentas), the digested material consisting of DNA bands of 30bp and 63bp was applied to a 12% polyacrylamide gel together with 6X loading buffer, and electrophoresed with 1X TAE buffer or 0.5X TBE buffer for 2h at room temperature, using 100V. Afterwards, the gel was stained for 30min with SYBR Green 1 (Sigma) diluted 10000 –fold with TAE or TBE. The digested DNA bands were detected by irradiating with UV rays at a wavelength of 250nm and their quantities were determined by the use of a dual-wavelength chromatoscanner. Together with 4 groups of recombinant marker viruses designated 472-a, 472-b, 472-c, 472-d, for which the percentage of 472-C values (determined by RI-MAPREC) were 0.72%, 1%, 2.8% and 4.0%, respectively, the calibration curve was prepared, in which the axis of abscissa indicates the fluorescence intensity of digested DNA bands and the axis of ordinate indicates the percentage quantities of 472-C. Using this calibration curve, we can determine the 472-C percentage of each single harvest based on the fluorescence intensity of digested DNA bands and decide whether it passes the MNVT or not.

For improved non RI-MAPREC applied in our laboratory, we compared the electrophoresis pattern of virus samples to the marker virus groups. A sample passes the test if no 63bp and 30bp digested DNA bands would appear or they appear but the bands are weaker than the corresponding bands of 472-b marker (472C percentage of 1%) - like the pattern of 472-a marker (472C percentage of 0.72%). For any case that is hard to compare by unaided eyes, the samples should be retested by *rct*40 test.

3. Results

Fig. 1 shows the pattern obtained by electrophoresis in analyzing type 3 polioviruses and 4 groups of recombinant marker viruses. All the vaccine samples produced at Poliovac in 2005 show no 30bp and 63bp bands while the corresponding bands of 472-b, 472-c, 472-d are obvious. The 63bp band of 472-a is hard to detect since its proportion of 472C is just 0.72%. All the type 3 single harvests pass the non RI-MAPREC and are safe for use.

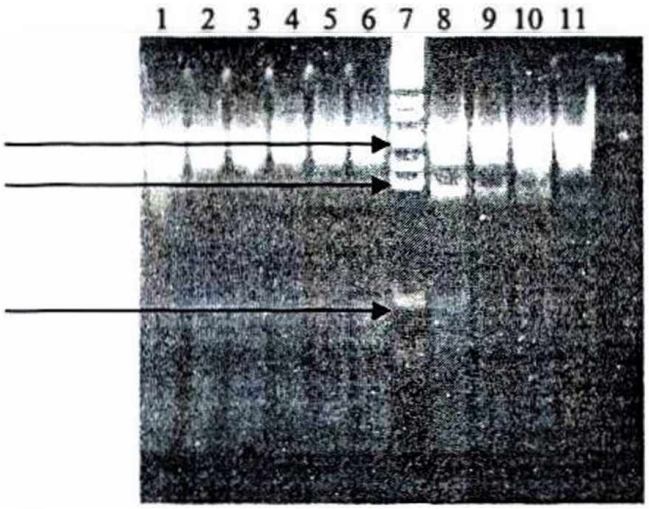

Fig. 1. Electrophoresis pattern of type 3 poliovirus by non RI-MAPREC with pBR322/*Msp*I ladder, arrows indicate 90bp, 67bp and 30bp bands. 1, 2, 3, 4, 5, 6, 7 are virus samples (single harvests) designated 402, 403, 404, 405, 406, 407, respectively. 8, 9, 10, 11 are 472-d, 472-c, 472-b and 472-a, respectively.

It therefore can be affirmed that MAPREC test is both effective and exact. We also compared this test to the usual *rct*40 test in terms of time consumed and expenses.

Table2. Comparison between the two tests in terms of time consumed and expenses (per 1 sample)

	Time consumed	Expenses
rct40 test	98h	~40USD
non RI-MAPREC test	25h	~10USD

So, compared in terms of time consumed and expenses, improved non-RI MAPREC test shows its undeniable advantages over *rct*40 test: much more cost-effective and less time-consuming. Therefore, it is highly recommended that this test should be used as an alternative to *rct*40 test for *in vitro* examination of poliomyelitis vaccine.

4. Discussion

A number of studies have established that changes in the nucleotide sequences of vaccine viruses can lead to the development of neurovirulent revertants [8,9]. Findings from studies of type 3 virus provide sound evidence that a single nucleotide change in the base at position 472 in the genome correlates directly with increased neurovirulence for monkey [6,9]. Our result demonstrates that the improved non RI-MAPREC test is useful for *in vitro* assessment of the safety of single harvests used to produce trivalent OPV.

Despite many advantages of non RI-MAPREC over RI-MAPREC as listed above, the detectable range of non-RI MAPREC is narrower than that achieved by MAPREC: in estimating the content of bases changes by non-RI MAPREC, for all three types of virus the upper limit is 15-20% (because the fluorescence intensity of digested bands is too strong to be measured) and the lowest limit is 0.3-0.4% (because the intensity is too thin to be measured). These comparative results demonstrate that MAPREC has the advantage of being able to detect a wider range of changes in nucleotide sequences than non-RI MAPREC. However, since the quantity of nucleotide change shown to confer neurovirulent properties to OPV is within the range of detectability of non-RI MAPREC, it is suggested that either procedure may be used to test OPV for neurovirulent properties [2]. Indeed, on the basis of practical conditions in our laboratory, the improved non RI-MAPREC test is really of great advantage.

The *rct*40 test is frequently used in our laboratory to assess the virulence of the vaccine viruses via their temperature sensitivity. The revertant is not temperature sensitive, therefore their reproductive capacity has no changes at the temperatures of 36°C and 40°C. Thus, by examining the reproductive capacity of vaccine viruses at different temperatures and comparing these results, we can indirectly assess the genetic material of the vaccine. However, nucleotide mutations that have the greatest contribution to virulence have only minor effect on viral temperature sensitivity. Therefore, if a vaccine sample shows temperature sensitivity, it does not mean totally that this sample is not neurovirulent, which is a great disadvantage of *rct*40 test that can be overcome by MAPREC. Moreover, compared in terms of time consumed and expenses, the improved non RI-MAPREC test shows its undeniable advantages over *rct*40 test.

These results, therefore, demonstrate that the improved non RI-MAPREC test should be used as an alternative to the usual *rct*40 test which has the disadvantages of being unavoidably costly and time-consuming, to supplement the MNVT. Genomic monitoring

by this method should prove to be important in order to confirm the consistency of vaccine viruses during production.

Acknowledgements

We are grateful to our colleagues in the QC department (Poliovac) and Dr Nguyen Dang Hien-director of Poliovac for their great support; Dr Dang Tuan Dat-Vaccine and Biological products company No1, Dr Nguyen Van Mui and Nguyen Quang Huy of College of Science, VNU as well as many scientists at Japan Poliomyelitis Research Institute for their great help of necessary chemicals and equipment; and also, we thank Dr. Pham Van Ty of College of Science for his invaluable comments.

References

[1] K.M. Chumakov, Mutant analysis by PCR and restriction enzyme cleavage (MAPREC) for oral poliovirus (Sabin) vaccine, Standard operating procedure, prepared for the WHO collaborative study on MAPREC, *USA. Version 3.2. January* 25 (1999).

[2] Hitoshi Horie, Yoshio Tano, Yutaka Doi, So Hashizume. Estimation of the neurovirulence of poliovirus by non – radioisotope molecular analysis to quantify genomic changes. *Biologicals*, USA.26 (1998) 289.

[3] B.M. Nkowane, S.G.F. Wassilak, W.A. Orenstein, K.J. Bart, L.B. Schonberger, A.R. Hinman, O.M. Kew, Vaccine – associated paralytic poliomyelitis, United States: 1973 through 1984, *JAMA*, USA. 257(1987)1335.

[4] Adrian McGoldrick, J. Andrew Macadam, Glynis Dunn, Alison Rowe, John Burlison, D. Philip Minor, Janet Meredith, J. David Evans, and Jeffrey W.Almond. Role of mutations G-480 and C-6203 in the attenuation phenotype of Sabin type 1 Poliovirus. *J.Virol*, USA. 69 (1995) 7601.

[5] D. Philip Minor, Poliovirus vaccination: current understanding of poliovirus interactions in humans and implications for the eradication of poliomyelitis, *Exp. Rev. Mol. Med online*, UK. September 23, 1999.

[6] Saumitra Das, Michael Ott, Akemi Yamann, Arun Venkatesan, Sanjeev Gupta, Asim Dasgupta, Inhibition of internal entry site (IRES)-mediated translation by a small yeast RNA: a novel strategy to block hepatitis C virus protein synthesis, *Frontiers in Bioscience*, USA. 3(1998) 1241.

[7] Maria-Magdalena, Georgescu, Maryse Tardy-Panit, Sophie Guillot, Radu Crainic and Francis Delpeyroux, Mapping of mutations contributing to the temperature sensitivity of the Sabin 1 vaccine strain of poliovirus. *J.Virol*, USA. 69 (1995) 5278.

[8] Kawamura N, Kohara M, Abe S *et al.*, Determinants in the 5' non coding region of poliovirus Sabin 1 RNA that influence the attenuation phenotype, *J.Virol*, USA. 63 (1989) 1302.

[9] G.D. Westrop, K.A. Wareham, D.M.A. Davis *et al.*, Genetic basis of attenuation of the Sabin type 3 oral poliovirus vaccine, *J.Virol*, USA. 163 (1989) 1338.

Sử dụng kỹ thuật Non-Ri Maprec cải biến đánh giá sự an toàn của Vaccine bại liệt uống giảm độc lực (OPV)-ưu thế so với thử nghiệm nhiệt độ *rct*40

Thiều Thị Hoài Anh, Lê Thị Luân

Trung tâm khoa học sản xuất vaccine Sabin (Poliovac), 135 Lò Đúc, Hà Nội

Kỹ thuật MAPREC – phân tích đột biến bằng kỹ thuật PCR và phân cắt bằng enzyme giới hạn (Mutant analysis by polymerase chain reaction and restriction enzyme cleavage) sử dụng đồng vị phóng xạ ^{32}P lần đầu tiên được đưa ra bởi Chumakov và cộng sự đã tỏ ra rất hữu hiệu trong việc đánh giá vật chất di truyền của virus bại liệt, cụ thể là những thay đổi trong cấu trúc genome có thể dẫn đến tính độc của vaccine bại liệt sống uống giảm độc lực (OPV). Sau đó, kỹ thuật MAPREC không sử dụng đồng vị phóng xạ (non RI-MAPREC) đã được phát triển dựa trên kỹ thuật MAPREC cơ bản nói trên bởi các nhà khoa học ở Viện nghiên cứu bại liệt Nhật Bản vào năm 1998 và đã tỏ rõ một số ưu thế so với MAPREC, giúp cho việc ứng dụng ở các nước đang phát triển như nước ta được dễ dàng hơn. Tại phòng thí nghiệm của chúng tôi, kỹ thuật non RI-MAPREC tiếp tục được cải biến để phù hợp hơn với điều kiện cụ thể của Việt Nam, đồng thời chúng tôi cũng so sánh để nêu ra những ưu điểm to lớn của kỹ thuật cải biến này với một thí nghiệm "cổ điển" vẫn được sử dụng trong phòng thí nghiệm để đánh giá sự an toàn của sản phẩm vaccine-thử nghiệm nhiệt độ *rct*40.