# Establishment of realtime RT-PCR assay to detect polio virus in the Acute Flaccid Paralysis laboratory surveillance

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## Abstrak

***Latar belakang:*** *Virus polio indigenous terakhir ditemukan di Indonesia tahun 1995 tetapi ancaman virus polio impor dan mutasi virus dari Oral Polio Vaccine (OPV) menjadi Vaccine Derived Poliovirus (VDPV) masih berlanjut. Tahun 1991 WHO mengembangkan Surveilans Acute Flaccid Paralysis (AFP) dan tahun 2014, identifikasi virus polio dengan real-time reverse transcriptase Polymerase Chain Reaction (rRT-PCR) mulai digunakan di Laboratorium Nasional Polio Pusat Biomedis dan Teknologi Dasar Kesehatan. Tujuan dari penggunaan rRT-PCR untuk mendapatkan metode yang cepat dan lebih baik dalam memantau sirkulasi dan mutasi virus polio.*

***Metode:*** *Isolat polio positif diidentifikasi menggunakanan rRT PCR dengan kombinasi primer dan probe yang ditetapkan WHO. RNA virus di konversi ke cDNA menggunakan reverse transcriptase lalu diamplifikasi menggunakan taq polymerase. Produk PCR di deteksi dan diidentifikasi dengan hibridisasi menggunakan probe spesifik. Sintesis cDNA dan reaksi PCR menggunakan primer yang dilekatkan di probe. Kombinasi primer dan probe menghasilkan identifikasi serotipe dan intratypic differentiation (ITD) dari isolat virus.*

***Hasil:*** *Selama tahun 2014, NPL Jakarta menerima 604 kasus AFP dari surveilans dan lima kasus terdeteksi positif mengandung virus polio. Semua spesimen positif mengandung virus polio yang berasal dari vaksin. Dua kasus positif virus polio tipe P2 (40%), satu kasus jenis virus polio P1 (20%), 1 kasus jenis virus polio P3 (20%) dan satu kasus virus polio campuran jenis P1 + P2 (20%).*

***Kesimpulan:*** *Real-time PCR dapat digunakan di Laboratorium Polio Jakarta untuk membantu identifikasi virus Polio secara cepat. Tes ini dapat digunakan untuk memantau sirkulasi virus polio pada populasi yang rutin diimunisasi dengan OPV.* ***(Health Science Journal of Indonesia 2016;7:27-31)***

***Kata kunci:*** *ITD, Poliovirus, Identification, rRT-PCR*

## Abstract

**Background:** The last indigenous polio was detected in 1995 but the threat of wild type polio viruses and the mutation of Oral Polio Vaccine into Vaccine-Derived Poliovirus still continue. Since 1991, WHO has developed Acute Flaccid Paralysis (AFP) laboratory based surveillance. In 2014, the polioviruses identification by real-time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR), has been introduced to National Polio Laboratory (NPL) Center for Biomedical and Basic Technology of Health. The objective of the rRT-PCR application is to have faster and better diagnostic methods to monitor the circulation and mutation of polio viruses.

**Methods:** Isolate tested by rRT-PCR using a combination of primers and probe mentioned by WHO manual. The viral RNA is converted to cDNA using reverse transcriptase and amplified in a PCR reaction using Taq polymerase. The PCR products are detected and identified by hybridization with specific probes. The combination of primers and probes will result in the serotype identification and intratypic differentiation of poliovirus isolates.

**Results:** In 2014 NPL Jakarta received 604 AFP cases through the surveillance system, five cases found positive for polio viruses by culture. All of the specimens were positive for polio vaccine viruses. Two cases were polio virus type P2 (40%), one cases polio virus type P1 (20%), 1 case polio virus type P3 (20%) and one case mix polio viruses type P1+P2 (20%).

**Conclusion:** The real-time PCR assay was able to help the identification of polio viruses rapidly in Jakarta lab. The test can be utilized for monitoring the population routinely immunized with OPV. ***(Health Science Journal of Indonesia 2016;7:27-31)***

**Keywords:** ITD, Poliovirus, Identification, rRT-PCR

Since 1988 the World Health Organization (WHO) has started global polio eradication program as mandated by the World Health Assembly. Since this program started, the progress has been made as the number of polio cases decreased globally and the number of countries which reported endemic transmission is reduced.[1-2] WHO has established an integrated virology surveillance network globally to monitor polio viruses transmission and circulation since 1990.[1] The objective of this network is to provide comprehensive epidemiology and virology data as a tool for public health measures toward polio eradication programs.[1] The monitoring of the poliovirus transmission through laboratory approach has been implemented in Indonesia since 1991 which previously based on clinical approach. Poliovirus monitoring was performed in cases with Acute Flaccid Paralysis (AFP) symptoms since 1995 as part of WHO global program. The integrative polio surveillance network in Indonesia was able to detect last wild-type poliovirus cases in 1995 and the polio importation cases in 2005.[3] Since South East Asia region had been certified as polio-free region in 2014, the virology surveillance network is still needed to monitor the circulation and transmission of polio virus from other non-polio free region.[4]

The Global Polio Laboratory Network (GPLN) consists of three levels; Global Specialized Laboratory, Regional Reference Laboratories, and National Laboratories.[1] Each levels has different responsibilities under the guideline set by WHO. In Indonesia, three laboratories have been designated as National Polio Laboratory (NPL) which act as national referral laboratory for poliovirus detection. Virology Laboratory Center for Biomedical and Basic Technology of Health (CBBTH), National Institute of Health Research and Development (NIHRD) acts as NPL for 16 provinces in the western part of Indonesia (all provinces on the island of Sumatra, Borneo and some in Java).[5]. As mentioned by the WHO polio laboratory manual, the main NPL activities is the virus isolation and serotype identification from AFP stool specimens received from national surveillance network.[1]

Viral isolation is the “Gold Standard” laboratory methods performed in all laboratories under GPLN. The observation of cytopathic effect in L20B and RD cells after virus isolation determine the presence of enterovirus in the specimens.[1] Poliovirus identification then can be performed using several methods such as neutralization, ELISA and hybridization. These methods also provide intratypic differentiation (ITD) between wild type and vaccine derived polio virus.[6,7] The ITD is performed by Regional Reference Laboratories (RRL).[1] Since 1999, the technique of Polymerase Chain Reaction (PCR) has been introduced to identify polioviruses from AFP specimens as part of the development of the polio identification methods that more accurate, quickly and reliable.[5] In 2014, the new diagnostic method technique of real-time reverse transcriptase polymerase chain reaction (rRT-PCR) for the identification of positive isolates polio has been introduced in NPL CCBTH Jakarta. The objective of this introduction is to have faster and better diagnostic methods which will help monitor the poliovirus circulation and mutation as NPL can perform ITD using these methods. This article will report the progress of PCR technique introduction in NPL Jakarta in 2014.

## METHODS

Stool specimens from AFP cases received from surveillance activities in 2014 were processed as WHO guidelines. Specimens were then inoculated into the Rhabdomyosarcoma (RD) and L20B cells. The grown virus in L20B cells than passage into RD cells. 50 µl of virus isolates grown in RD cells were spun at 5000 rpm for 2 minutes at room temperature.

The RT-PCR method used in this procedure was described previously.[8] The supernatant (0.5 µl) and 1 µl of control RNA as a positive control was added into each well or 0.2 ml tube strip were already contains a reagent which consists of 19 µl of buffer A (primers and probes) and 5 µl of buffer B has been added enzymes (2.8 mL of 1 M DTT, 27.6 mL of 40 U/ml RNase inhibitor, 18.0 mL of 20 U/ml transcription RT (14.4 mL or 25 U/ml AMV RT) in 1 ml of buffer B). one well/tube strips used as a negative control without added any template. The programs that used for PCR were 42°C, 45 min for RT reaction, 95°C, 3 min for inactivation of RT (holding stage) and with dual-stage PCR cycles.

**RT PCR for ITD**. This method had two-stages. Stage 1 of 15 cycles with the condition of 95°C for 24 seconds, 44°C for 30 seconds, then 25% ramp speed and 65°C for 24 seconds. Followed by stage 2 of 40 cycles with the condition of 95°C for 24 seconds, 47°C for 30 seconds, then 25% ramp speed and 65°C for 24 seconds. Data were collected at the end of stage 2. If the results are expressed positive ITD sabin (SL) / vaccine as CT value between 10-28, the curves should be compared with positive and

negative control. After this step, it would be followed by VDVP test.

**RT PCR for VDVP**. This method is similar to the ITD but differs in primer and probe in buffer A and its PCR cycles. A PCR cycle on VDVP test also has a dual stage. Stage 1 by 5 cycles with the condition of 95°C for 24 seconds, 44°C for 30 seconds and 60°C for 24 seconds followed by a second stage of 40 cycles with the condition of 95°C for 24 seconds, 50°C for 30 seconds, and 25% ramp speed and 65°C for 24 seconds. If the result is positive (CT value 10-28) then the specimens were concluded as a positive for polio virus from the vaccine. If the result is negative then it will be proceed with the sequencing.[8]

## RESULTS

Table 1 shows the number of AFP cases reported and the number of polio and non polio enterovirus (NPEV) positive by cultured every month. Fortunately, isolation of virus from stool extracts also cultured other enteroviruses/ NPEV. From January to December 2014 the National Polio Laboratory CBBTH NIHRD Jakarta received 604 cases of AFP surveillance with 1227 specimens and only 1% of AFP cases of AFP were positive of poliovirus and 8% were positive of NPEV in the stool.

Table 2 shows the distribution of poliovirus and NPEV positive cases by age group and gender. The polio and NPEV positive cases in the age group 1-5 is higher than the other age group.

The positive isolates were identified using realtime PCR methods. Tabel 3 shows all of the polio viruses from AFP cases were sabin like and polio virus type 2 (P2).

## DISCUSSION

This study conducted on the specimens from AFP cases sent to NPL CBBTH during 2014. The results showed that not all AFP cases are caused by poliovirus, only 1% of AFP cases of AFP were positive of poliovirus. This result is similar to the result from previous research that Guillain Barre Syndrome is the most common cause in the AFP case.[9,10] The results in several National Laboratories worldwide also showed that NPEV from AFP cases were quite high.[11]

Identification for poliovirus at AFP cases need more rapid and sensitive method. Realtime RT-PCR method more objective and faster than previous methods (neutralization test and hybridization) which takes a more time and need experience and skill.[12] Identification of poliovirus using Realtime RT-PCR method is performed on L20B positif cell cultures that show characteristic Cytopathic Effect (CPE).[1]

Realtime RT-PCR method utilizes multiple sets of oligonucleotide primers combined with probes to identify the serotypes and intratypic differentiation of poliovirus. This method can detect the presence of NPEV and poliovirus that mixture in the isolation medium. Realtime RT-PCR for poliovirus identification is designed with degenerate primer by targeting particular amino acid for each serotype of polioviruses. Degenerate primers are designed to compensate error due to nucleotides synthesized.[8]

Table1.Distribution ofpolioAFP casesand Positive

| Month | No of AFP Cases | No of Specimens | No. of Polio Positive Specimens | No. of Polio Positive Specimens polio positive by PCR | No. of NPEV Positive Specimens |
|---|---|---|---|---|---|
| January | 30 | 62 | 0 ( 0% ) | 0 ( 0% ) | 2 ( 3% ) |
| February | 52 | 108 | 0 ( 0% ) | 0 ( 0% ) | 5 ( 5% ) |
| March | 53 | 107 | 1 ( 2% ) | 2 ( 2% ) | 13 (12%) |
| April | 54 | 113 | 2 ( 4% ) | 4 ( 4% ) | 10 ( 9% ) |
| May | 56 | 113 | 1 ( 2% ) | 2 ( 2% ) | 16 (14% ) |
| June | 40 | 80 | 1 ( 3% ) | 2 ( 3% ) | 8 (10% ) |
| July | 31 | 62 | 0 ( 0% ) | 0 ( 0% ) | 6 (10% ) |
| August | 26 | 50 | 0 ( 0% ) | 0 ( 0% ) | 1 ( 2% ) |
| September | 42 | 86 | 0 ( 0% ) | 0 ( 0% ) | 2 ( 2% ) |
| October | 75 | 153 | 0 ( 0% ) | 0 ( 0% ) | 11 ( 7% ) |
| November | 74 | 152 | 2 ( 3% ) | 4 ( 3% ) | 14 ( 9% ) |
| December | 71 | 141 | 0 ( 0% ) | 0 ( 0% ) | 9 ( 6% ) |
| **Total** | **604** | **1227** | **7 ( 1% )** | **14 ( 1% )** | **97 ( 8% )** |

Table 2. Demographic distribution of positive Polio and NPEV cases

| Characteristics | No of Cases | No of Polio Positive (%) | No of NPEV Positive (%) |
|---|---|---|---|
| Age group (years old) | | | |
| 0 – 1 | 49 | 3 (6.1) | 10 (20.4) |
| > 1 – 5 | 298 | 3 (1) | 60 (20.1) |
| > 5 – 15 | 257 | 1 (0.4) | 27 (10.5) |
| Sex | | | |
| Male | 326 | 4 (1.2) | 66 (20.2) |
| Female | 278 | 3 (1.1) | 31 (11.1) |

Table 3. Serotypes of Poliovirus from AFP cases

| Serotype | No. of Positive (%) |
|---|---|
| P1 Sabin Like | 2 (14) |
| P1+P2 Sabin Like | 2 (14) |
| P2 Sabin Like | 8 (57) |
| P3 Sabin Like | 2 (14) |
| Total | 14 (100) |

There are three types of polio virus circulating which are Sabin, VDPV and wild type. Sabin virus is obtained from the polio vaccine and as the virus can mutate in non-vaccinated or immunocompromised leading to VDPV with a 1% difference in nucleotide Sabin reference. (REF) Currently, the wild polio virus was found only in the country of Pakistan, Afghanistan and Nigeria. (REF)

Poliovirus might infect people at any age and gender with the children under five years is more susceptible to the infection. The children in the age group 1-5 years are susceptible than under 1 year because they are more active than the children under 1 year old. Moreover, the maternal immunity is assumed to protect the children under 1 year from poliovirus infection.[13,14]

Identification of poliovirus using Realtime RT-PCR method on three types of poliovirus ~~found~~ in the stool specimens from positive cases as showed in Table 3 and P2. Sabin was the more common serotype (60%). The use of oral trivalent poliovirus vaccine (OPV) in Indonesia might cause the presence of poliovirus in stools because the virus still replicates in the intestine for several weeks after vaccination and then are excreted in the feces. There is competition among the three type viruses in the development of immunity. OPV is most effective against type 2. The type 2 virus from OPV replicated in intestine and is excreted in the stool. [1,15]

**In conclusion,** the real-time PCR assay was able to help the identification of polio viruses rapidly in Jakarta lab. The test can be used for monitoring the mutation and circulation of poliovirus.

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