Video Article

Particle Agglutination Method for Poliovirus Identification

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Abstract

In the Global Polio Eradication Initiative, laboratory diagnosis plays a critical role by isolating and identifying PV from the stool samples of acute flaccid paralysis (AFP) cases. In the World Health Organization (WHO) Global Polio Laboratory Network, PV isolation and identification are currently being performed by using cell culture system and real-time RT-PCR, respectively. In the post-eradication era of PV, simple and rapid identification procedures would be helpful for rapid confirmation of polio cases at the national laboratories. In the present study, we will show the procedure of novel PA assay developed for PV identification. This PA assay utilizes interaction of PV receptor (PVR) molecule and virion that is specific and uniform affinity to all the serotypes of PV. The procedure is simple (one step procedure in reaction plates) and rapid (results can be obtained within 2 h of reaction), and the result is visually observed (observation of agglutination of gelatin particles).

Protocol

1. Preparation of maintenance medium (MM) and growth medium (GM)

For preparation of MM and GM, please see a Polio laboratory manual of WHO¹. Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal calf serum (FCS) and 10% FCS could be used as substitutes of MM and GM, respectively.

2. Preparation of anti-PV antibodies

1. Reconstitute anti-PV antibody (RIVM PV typing antiserum) by adding water (0.5 mL per vial) to each vial of anti-PV antibody (type 1, 2, or 3). Add this reconstituted antibody solutions (0.5 mL each) to 4.5 mL of MM in separate labeled tubes.
Note: Some lots of anti-PV antibody are provided as reconstituted form (0.5 mL per vial). For such lot of anti-PV antibody, addition of water for reconstitution is not necessary.
2. Mix equal volume of reconstituted anti-PV antibody. For anti-PV1 +2, 2+3, or 3+1: Mix 1.8 mL of each reconstituted antibody (1.8 mL + 1.8 mL = 3.6 mL for each mixture). For anti-PV1 +2+3: Mix 1.2 mL of each reconstituted antibody (1.2 mL + 1.2 mL + 1.2 mL = 3.6 mL).
Note: PV samples could contain a mixture of different serotypes of PV. To identify single serotype of PV in samples with mixture of PVs, pools of anti-PV antibodies (i.e. 1+2, 2+3, 3+1, 1+2+3) is necessary.
3. Add 1/5 volume of FCS (0.72 mL) to each anti-PV (1+2, 2+3, 3+1, or 1+2+3) antibody (3.6 mL each) (final 18.2% FCS).
Note: Addition of FCS is for reduction of non-specific agglutination of gelatin particle caused by antibodies.
4. Keep the antibody pools at -20 °C until use in PA assay.

3. Reconstitution of sensitized gelatin particle

1. Add 1.5 mL of reconstitution buffer included in the kit to one vial of sensitized gelatin particle (provided as freeze-dried powder).
2. Mix well by gentle tapping
3. Keep the reconstituted sensitized particle at 4 °C. Reconstituted gelatin particle could be active at least 2 weeks kept at 4 °C, but should be prepared just before the assay.

Important note:

Do not mix different lot of sensitized gelatin particles, because the non-agglutination image of sensitized gelatin particles could be different by the lot of the particles. For each experiment, a single lot of sensitized gelatin particle should be used.

4. PA assay for PV identification

1. Prepare a micro-titration plate. For identification of one virus sample, we need 5 wells + at least one negative control (sensitized particle without virus and antibody, well # 6 in below. See No virus and antibody control in Example). Total number of wells is 5 x samples + at least one control in the assay.
Important note:
Please take at least one No virus and antibody control in the assay. One vial of sensitized particle is approximately for identification of 10 virus samples.
2. Add 12 µL/well of anti-PV antibody or GM to the micro-titration plate. The arrangement of the plate is as follows (See also Example below). In samples without antibody (well #1 and 6), add 12 µL of GM instead of anti-PV antibody.
Important note:

If many PV samples should be tested in this assay, multichannel pipette or dispenser would be helpful for this step.

Well #1	Well #2	Well #3	Well #4	Well #5	Well #6
GM	anti-P1+2+3	anti-P1+2	anti-P2+3	ani-P1+3	GM

- Add 12 μ L/well of virus sample. In one sample without virus (well #6), add 12 μ L of GM instead of virus solution.

Note: Virus sample is culture supernatant of infected cells, usually L20B cells or RD cells.

Important note:

If many PV samples should be tested in this assay, multichannel pipette would be helpful for this step.

Well #1	Well #2	Well #3	Well #4	Well #5	Well #6
virus sample	virus sample	virus sample	virus sample	virus sample	GM

- Take reconstituted sensitized particle solution from 4 °C to room temperature, and gently tap to completely suspend the particles.
Important note:
After complete suspension of the particles by gentle tapping, the particles should be added immediately to the plates before the suspended particles precipitate and form non-homogeneous solution.
- Add 25 μ L/well of sensitized particle to all the wells.
Important note:
After addition of sensitized particle, the plate should immediately be subjected to plate mixing to start uniform reaction of the samples. Therefore, addition of sensitized particle should be performed by using dispenser to minimize a time lag among the samples in the plate.
- Put the micro-titration plate onto a plate mixer and mix the plate for 30 sec.
- Put the micro-titration plate on white paper (for convenience of visual observation) on a table at room temperature (15~30 °C) for 2 h.
Important note:
Do not move the plate during and after the reaction for observation of the results and taking photos. The formed agglutination is not stable and easily disturbed by moving the plates.
- Take photos of the plate.
Important note:
Partial view of the photos could not give sufficient data for later examination and review of the results for interpretation. Several photos should be taken to cover all the wells of the plates focusing on the agglutination image of the wells.

5. Interpretation of the results

- Identification of positive and negative wells. Compare the agglutination in the samples with that of No virus and antibody control (precipitation of gelatin particles, non-agglutination) (Figure 1 and 2). Images of agglutination that are clearly distinguished from No virus and no antibody control (non-agglutination, negative control) should be judged as positive, and images of agglutination that are similar to the negative control should be judged as negative. Samples that show clear but only partial agglutination could be judged as positive, but record as 'Positive (partial agglutination)'.
- Identification of PV. Interpret the results based on positive and negative wells with anti-PV antibodies to identify PV serotypes. Principle of the interpretation of the result is similar to that of neutralization test in cell culture system (e.g. positive for anti-P1+2 antibody indicates that the sample contains type 3 PV).

6. Representative Results

Representative result of PA assay PV identification is shown in **Figure 1**. All the PV strains formed agglutination of sensitized gelatin particles (No antibodies samples). In the presence of corresponding anti-PV antibodies, precipitation of gelatin particles (non-agglutination) similar to No virus and no antibodies control (negative control) was observed (e.g., PV1(Sabin) in the presence of anti-P1+2 or anti-P3+1 antibodies).

Figure 1. Identification of PV by PA assay. Virus solutions of PV1(Sabin), PV2(Sabin), and PV3(Sabin) (virus titers of 1.2×10^8 CCID₅₀ / 12 μ L, 8.2×10^7 CCID₅₀ / 12 μ L, and 3.5×10^7 CCID₅₀ / 12 μ L, respectively) were used for the identification by PA assay. No virus and no antibodies control showed precipitation of gelatin particles (non-agglutination, Negative control).

Figure 2. Interpretation of Positive and Negative wells. Virus solutions of PV1(Sabin), PV2(Sabin), and PV3(Sabin) (virus titers of 2.4×10^8 CCID₅₀ / 25 μ L, 1.7×10^8 CCID₅₀ / 25 μ L, and 7.3×10^7 CCID₅₀ / 25 μ L, respectively) were diluted 1/20 to 1/320, and then 25 μ L of diluted samples were examined by PA assay without anti-PV antibodies.

Discussion

The unique point of this PA assay is the utilization of PVR molecule instead of anti-PV antibodies for the detection of PV 2. This allows a specific interaction of the sensitized gelatin particles with PV with a uniform affinity to all three serotypes of PV ³⁻⁶. We used an immunoadhesin form of PVR (PVR-IgG2a) for the sensitization of gelatin particles, because monomeric forms of PVR have only moderate affinities to the single binding site on the virion (dissociation constant of 10^{-7-8} M) ^{7,8}, and successfully observed specific agglutination of the sensitized gelatin particles with PV isolates. With the high specificity and uniform affinity of the sensitized particles, the critical factors of PV identification in the PA assay are 1) virus titer (for valid detection of PV by PA assay) and 2) interpretation of agglutination (for correct identification of PV).

Generally, virus titer of PV isolates in the cell culture system is in a range of 10^6 to 10^8 CCID₅₀ / 50 μ L. The detection limits of PA assay (forming complete agglutination) for type 1, 2, and 3 PV were 1.5×10^6 CCID₅₀, 5.3×10^5 CCID₅₀, and 9.1×10^5 CCID₅₀, respectively ². The PA assay correctly identified most of the PV isolates even in the presence of other non-PV enteroviruses. However, for some samples contained a mixture of different serotypes of PV, minor serotype failed to be detected. Clear agglutination should be formed with sufficient virus titer above the detection limits. In case the agglutination in No antibodies sample was unclear or partial, the isolates should be re-amplified in RD cells to increase the titer ⁹.

Partial agglutination was observed when the virus titer was low and just below the detection limits. Clear difference of agglutination from negative control could be judged as positive in PA assay. However, quite subtle difference of the agglutination judged as positive could mislead the interpretation of results. Therefore, the results of Positive (partial agglutination) should be used as supportive information for possibly caused by minor serotypes of the isolates. However, this information would be helpful when following analysis (genomic sequencing of PV isolates or real-time RT-PCR) suggested a possibility that the sample contained a mixture of different serotypes of PV, or only partial agglutination was observed for No antibodies sample where the low virus titer would be the major cause of the unclear results.

The advantage of PA assay for PV identification is in the simplicity of the procedure and result interpretation. This would allow easy introduction of the assay to every laboratories conducting PV isolation with the cell culture system, without providing special equipment, materials, and trainings. Utilization of type-specific monoclonal antibodies might allow the development of novel method for the differentiation of PVs (wild type or vaccine-like strains) that would provide important information of the property of the PV isolates along with genetic differentiation by RT-PCR and genomic sequencing ^{10,11}.

Disclosures

Souji Masujima is an employee of New Product Design Department, Fujirebio Inc.

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