Chapter 7

A Transgenic Mouse Model of Poliomyelitis

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Abstract

Transgenic mice (tg mice) that express the human poliovirus receptor (PVR), CD155, are susceptible to poliovirus and develop a neurological disease that resembles human poliomyelitis. Assessment of the neurovirulence levels of poliovirus strains, including mutant viruses produced by reverse genetics, circulating vaccine-derived poliovirus, and vaccine candidates, is useful for basic research of poliovirus pathogenicity, the surveillance of circulating polioviruses, and the quality control of oral live poliovirus vaccines, and does not require the use of monkeys. Furthermore, PVR-tg mice are useful for studying poliovirus tissue tropism and host immune responses. PVR-tg mice can be bred with mice deficient in the genes involved in viral pathogenicity. This report describes the methods used to analyze the pathogenicity and immune responses of poliovirus using the PVR-tg mouse model.

Key words Poliovirus receptor, Rodent model, Neurovirulence, Intracerebral inoculation, Paralysis, Viral antigen, Motor neuron, Innate immune response, Knockout mouse, Tissue tropism

1 Introduction

Humans are the natural hosts of poliovirus, but chimpanzees and old-world monkeys are also susceptible to experimental infections with poliovirus. In general, other animal species are not susceptible to poliovirus, except for some artificially adapted virus strains [1–3]. This narrow host range means that monkeys have been the main model for studying poliovirus infection in vivo. However, experiments that use monkeys are not only very expensive and labor intensive but also entail ethical problems and a potential risk of zoonoses.

Murine cells are resistant to poliovirus infection; however, transfection of viral RNA into these cells yields infectious particles, which suggests that poliovirus can complete its replication cycle even in mouse cells, provided the virus can circumvent the entry step [4–6]. The viral receptors for picornaviruses play important roles in attachment, internalization, and uncoating of poliovirus [7]. These previous studies, and other experimental evidence,

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suggest that the poliovirus receptor (PVR) is the primary determinant that restricts the host cell tropism. The "*Poliovirus sensitivity* (*PVS*)" gene is located on human chromosome 19 [8]. Mendelsohn et al. [9] showed that the lack of susceptibility of mouse L cells was overcome by the ectopic expression of a gene of human origin. This gene was PVR, which encodes an integral membrane protein comprising three immunoglobulin-like domains within the extracellular region [10, 11]. The NH_2-terminal Ig-like domain of PVR binds the virus, whereas the mouse ortholog or paralogs of PVR, i.e., tumor-associated glycoprotein-E4 (Tage4) and poliovirus receptor-related protein-1 and -2 (Prr-1 and Prr-2), cannot [12–16].

Thus, previous studies strongly suggest that transgenic expression of the human *PVR* gene will render mice susceptible to poliovirus infection. Ren et al. [17] and Koike et al. [18] introduced cosmid DNAs, which encoded the whole human *PVR* gene, into mice; PVR was then expressed under the control of its own promoter. The PVR protein was expressed in a wide range of cells in the transgenic mice (tg mice). After inoculating poliovirus via the intracerebral, intravenous, and intraperitoneal routes, the mice developed a paralytic disease that resembled human poliomyelitis. However, the mice were not as sensitive as humans to oral infection. Histopathological examination of the paralyzed mice showed that neurons in the central nervous system were infected and destroyed by poliovirus. No severe pathological changes were observed in other organs.

Oral live Sabin vaccines were developed from parental virulent strains after serial passage in cultured cells [19]. The molecular mechanism underlying attenuation has been one of the most important issues in poliovirus research. Nucleotide sequence analysis showed that the attenuated Sabin 1 strain harbored 57 nucleotide and 21 amino acid substitutions compared with the parental Mahoney strain [20]. The neurovirulence levels of the two viruses and their recombinants were evaluated using monkeys, and several determinants of the attenuation phenotype were mapped in the viral genome [21, 22]. Similarly, determinants of the attenuation phenotypes were identified in type 2 and 3 polioviruses [23, 24]. The most important common genetic determinant in the three serotypes was a nucleotide substitution in stem-loop V of the internal ribosomal entry site (IRES) within the 5′ noncoding region. The substitution in the IRES modifies the stem-loop structure and reduces the translation initiation efficiency of the poliovirus protein [25, 26].

Initially, it was not clear whether the neurovirulence levels of viruses evaluated using the tg mouse model correlated with those obtained using monkey models. Thus, the recombinant viruses were inoculated into PVR-tg mice [27–29] and the neurovirulence level of each virus was evaluated based on the 50 % lethal dose

(LD_{50}) or the 50 % paralysis dose (PD_{50}). The neurovirulence levels determined using the tg mouse model correlated well with those using the monkey model. This suggested that it is possible to use tg mice for neurovirulence tests instead of monkeys. The evaluation of monkey neurovirulence levels requires precise histopathological examination; however, the mice died or were paralyzed in a dose-dependent manner after poliovirus inoculation. Therefore, it is possible to evaluate the neurovirulence levels in tg mice simply by calculating the LD_{50} or PD_{50}, without the need for histological examination [29, 30]. Of the PVR-tg mouse lines, PVR-tg21 was selected for further study because its sensitivity range was suitable for the evaluation of attenuated and virulent virus strains [28, 31]. The tg mice are more sensitive to intraspinal inoculation of poliovirus than to intracerebral inoculation, which means that intraspinal inoculation is suitable for the validation of oral live polio vaccine lots. This method is described in detail in previous studies [29, 32]. The intracerebral inoculation route is suitable for evaluating relatively virulent strains. PVR-tg mice have been used to evaluate the circulating vaccine-derived polioviruses, which caused recent outbreaks [33–37].

PVR-tg mice have also been used to study the tissue tropism and host immune responses of poliovirus. Mechanisms of host innate immune response have been elucidated in the last decade and mice deficient in the genes involved in the responses have been generated. Initially, the roles of these genes were investigated using viruses that can infect mice. Crossing these knockout mice with PVR-tg mice allowed similar studies to be conducted using poliovirus infections. PVR-tg21 mice were first generated on an ICR background. ICR is not an inbred strain, so the mice were not suitable for certain types of studies, such as the immunological responses to poliovirus infection. A number of knockout mouse strains were maintained on a C57Black/6 (B6) background. Thus, the tg mice were backcrossed with B6 for more than ten generations, and then crossed with knockout mouse strains on a B6 background. Poliovirus preferentially replicates in neurons in the central nervous system (CNS), although the reason for this tissue tropism remains unknown. Thus, PVR-tg mice lacking the interferon (IFN) alpha/beta receptor 1 (*Ifnar1*) gene were highly susceptible to poliovirus infection [38]. Interestingly, poliovirus antigens were detected in the liver, spleen, and pancreas, sites at which viral antigens are seldom found in mice showing a normal IFN response. These results suggested that the innate immune response mediated by type I IFN plays an important role in protecting organs that are not targets of poliovirus. Thus, it was concluded that the IFN response controls the pathogenicity and tissue tropism of poliovirus.

Viral infections are sensed by receptors for pathogen-associated molecular patterns. Abe et al. and Oshiumi et al. investigated the important receptor-signaling pathways that sense poliovirus infection.

They crossed PVR-tg mice with *Rig-I*, *Mda5*, *Tlr3*, *Tlr7*, *Trif,* and *Myd88*-deficient mice and found that the TLR3-TRIF pathway plays a pivotal role in host innate immune responses [39, 40]. The generation of PVR-tg mice lacking other genes will help us to further understand the roles of genes involved in viral replication and host defense. The present report describes the following methods: (1) mouse neurovirulence testing via intracerebral inoculation; (2) the genotyping of PVR-tg mice; and (3) the detection of poliovirus antigens in PVR-tg mice. These techniques have been used to study the pathogenicity and immune responses to poliovirus in PVR-tg mice.

2 Materials

2.1 Mouse Neurovirulence Test of Poliovirus via Intracerebral Inoculation

1. A needle for intracerebral inoculation (size 27G × 2 mm tip, 25G × 10 mm neck) (Fig. 1). Alternatively, an intradermal disposable needle (size 27G × 3 mm tip, 25G × 10 mm neck) can be used.
2. A 0.25 mL glass syringe.
3. Mice: PVR-tg 21 mice on a B6 background (B6-PVR-tg21 mice can be obtained from Tokyo Metropolitan Institute of Medical Science) or an IQI background (IQI-PVR-tg21 mice are sold by CLEA, Japan).

2.2 Genotyping Protocol for the PVR Gene

1. Lysis buffer: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.45 % (v/v) Nonidet P40, 0.45 % (v/v) Triton X-100, 0.1 % (w/v) gelatin. Sterilize by autoclaving and store the lysis buffer at −20 °C in aliquots of 4 or 8 mL.
2. Proteinase K (recombinant PCR grade): Dissolve the Proteinase K powder in 20 mL of 1 M Tris–HCl (pH 8.3) (final concentration of 5 mg/mL) and store the solution in 100 μL aliquots at −20 °C.

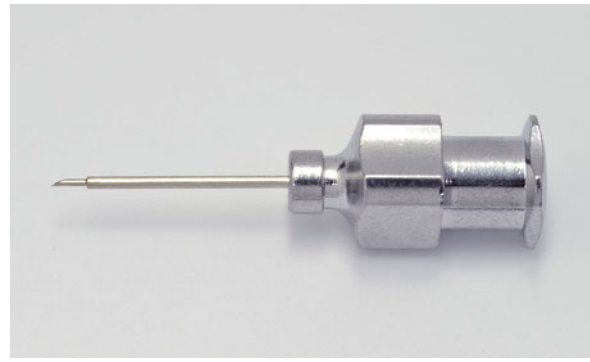

Fig. 1 Needle used for intracerebral inoculation. The needle comprises two parts, i.e., a 27G × 2 mm tip and a 25G × 10 mm thick neck, which helps to stop the needle at the desired depth

3. Primers:

PVR-4F	5′-ACCCCCCAGAGGTATCCATCTC-3′
MPH-4R	5′-TCATAGTCTGTGGGCTCTGGGTT-3′
IFNAR-51	5′-AGACGAGGCGAAGTGGTTAAAAGT-3′
IFNAR-32	5′-CTGTGTAGAATGGAATAAACGGATCA-3′
neo-N3	5′-GAACACGGCGGCATCAGAGC-3′

4. Thermostable *Taq* DNA polymerase.
5. ×10 PCR buffer: 100 mM Tris–HCl (pH 8.3), 500 mM KCl.
6. Restriction enzyme *Msp*I.
7. ×10 M buffer: 100 mM Tris–HCl (pH 7.5), 500 mM NaCl, 100 mM $MgCl_2$, 10 mM dithiothreitol.

2.3 Detection of Poliovirus Antigens

1. Butterfly catheter (22G).
2. Formalin-fixed, paraffin-embedded tissue.
3. Precoated glass slides.
4. Coplin jars or staining containers.
5. Autoclave.
6. Humidified chamber.
7. Coverslips.
8. Light microscope.
9. Fixative solution: Freshly prepared 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (PFA).
10. Decalcifying solution: Ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetrasodium salt, tetrahydrate (EDTA-4Na) 100 g, citric acid monohydrate 15 g, phosphate-buffered saline (PBS) to make up to 1 L.
11. Wash solution: 10 mM phosphate buffer (pH 7.2–7.3) containing 150 mM NaCl.
12. Antigen retrieval regents: 10 mM citric acid buffer (pH 6.0).
13. Chromogen substrate: DAB (3,3′-diaminobenzidine) substrate solution in 0.1 M Tris–HCl buffer (pH 7.6). (Add 100 μL of H_2O_2 to 150 mL of DAB solution in Tris–HCl buffer immediately before performing the chromogen substrate reaction.)
14. Antibody diluent: 0.1 % bovine serum albumin in PBS. To preserve the antibodies, add 0.01 % NaN_2 to the antibody diluent.
15. Graded ethanol series, i.e., 70, 80, 95, and 100 % ethanol.
16. Xylene.
17. 3 % hydrogen peroxide in methanol.

18. Primary antibody: Rabbit anti-poliovirus serum.
19. Secondary antibody: HRP-labeled polymer conjugated to secondary antibodies.
20. Distilled water.
21. Hematoxylin counterstaining reagent.

3 Methods

3.1 Mouse Neurovirulence Test of Poliovirus via Intracerebral Inoculation

1. Prepare 6- to 7-week-old PVR-tg mice. Ten (≥6) mice (the same number of each gender) are recommended for inoculation with each dose (*see* **Note 4.1-1**).
2. Dilute the virus stock in Eagle's Minimum Essential Medium (MEM) and prepare a serial tenfold dilution range from 10^1 to 10^7 plaque-forming units (PFU)/25 μL (*see* **Note 4.1-2**).
3. Anesthetize the mice via the inhalation of 4 % isoflurane. The use of anesthesia apparatus is recommended (*see* **Note 4.1-3**).
4. The mouse is restrained manually on a solid surface. Disinfect the head of the mouse with 70 % ethanol and inoculate 25 μL of virus solution (for each dilution) into the left hemisphere of the brain. The site of injection is approximately halfway between the eye and the ear, and just off the midline (*see* **Note 4.1-4**).
5. Confirm that the mice can walk without any problems after recovering from the anesthetic (*see* **Note 4.1-5**).
6. Keep the mice in an isolator rack and observe daily for 14 or 21 days to monitor paralysis and death.
7. Score the LD_{50} or PD_{50} values according to the formula of Kaerber [41] or Reed and Muench [42] (*see* **Note 4.1-6**).

3.2 Genotyping Protocol for the PVR Gene

A description of the strategy used to establish the *PVR*$^{+/+}$ *Ifnar1*$^{-/-}$ mouse strain is provided as an illustrative example. First, PVR-tg mice are crossed with *Ifnar1*$^{-/-}$ mice [43]. When the resulting F1 mice (*PVR*$^{+/-}$ *Ifnar1*$^{+/-}$) are ≥7 weeks old, they are bred with each other to obtain F2 mice. The progeny F2 mice comprise nine different genotypes, including *PVR*$^{+/+}$ *Ifnar1*$^{-/-}$. However, the general PCR protocols for detecting the transgene cannot discriminate between the *PVR*$^{+/-}$ and *PVR*$^{+/+}$ genotypes. Therefore, to select *PVR*$^{+/+}$ mice, it is necessary to amplify the exon 4 region of the human *PVR* gene and the homologous mouse PVR-related gene-2 (*Prr-2*, which is also referred to as *Nectin-2* and *Mph*) simultaneously using a set of PCR primers. The hPVR and mPrr-2 fragments can then be discriminated after *Msp*I digestion because the Prr-2 fragment contains an *Msp*I site. After comparing the band intensities of the hPVR and mPrr-2 fragments, it is possible to identify

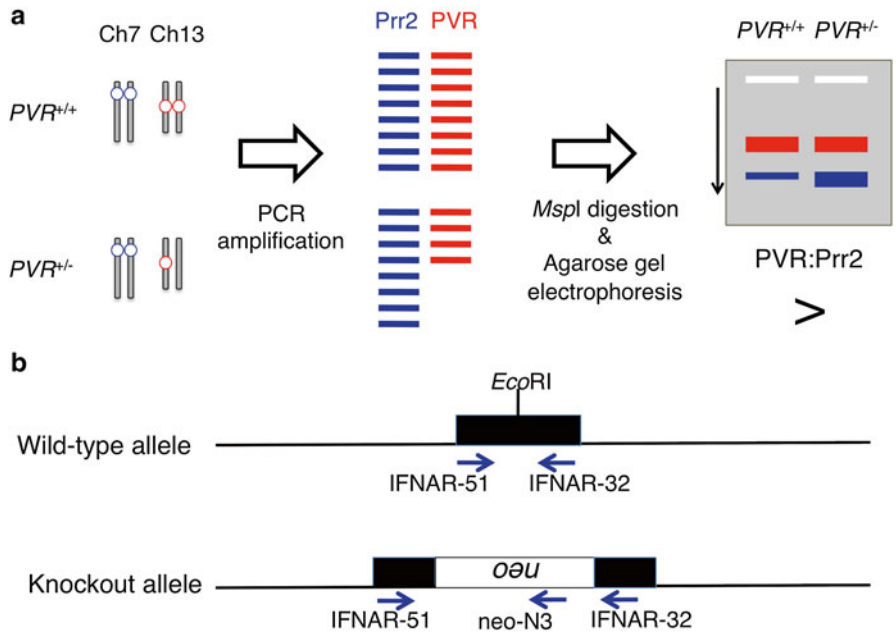

Fig. 2 Method used for identifying the *PVR* genotype (**a**) and the *Ifnar1* genotype (**b**) by PCR. (**a**) The *PVR* transgene (*red circle*) and the mouse *Prr2* gene (*blue circle*) are located on mouse chromosomes 13 and 7, respectively. The DNA ratio of PVR:Prr2 in the genomes of homozygotes is twice that in hemizygotes. The PVR and Prr2 fragments are co-amplified using a common primer. The *red* and *blue bars* represent PVR and Prr2 gene fragments, respectively. The ratio of the amplified fragments reflects the initial ratio. The length of both fragments is 109 bp, but only the mouse Prr2 fragment contains an *Msp*I site and can be cleaved into 87 and 22 bp fragments. The intensity of PVR fragment is higher than that of the Prr2 fragment in the $PVR^{+/+}$ genotype, whereas the intensity of the two fragments is comparable in the $PVR^{+/-}$ genotype. (**b**) The *Ifnar1* gene is disrupted by inserting the *neo* gene at the *Eco*RI site in the exon 3 region. The primers IFNAR-51 and IFNAR-31 anneal close to the 5′- and 3′- ends of exon 3 of the *Ifnar1* gene, respectively. Neo-N3 anneals to the *neo* gene. The wild-type Ifnar1 fragment is amplified using IFNAR-51 and IFNAR-32 primers (178 bp), and the Ifnar1-neo fusion fragment is amplified using IFNAR-51 and neo-N3 primers (465 bp)

individuals with the $PVR^{+/+}$ genotype (Fig. 2a). To select the $Ifnar1^{-/-}$ genotype, it is necessary to discriminate between the wild-type allele and the targeted allele using PCR with three different primers (Fig. 2b).

1. Cut a small piece (approximately 1 mm length) of tail from each F2 progeny mouse and place in a 1.5 mL sample tube. At the same time, make nicks in the ears of the mice to distinguish the individuals (*see* **Note 4.2-1**).
2. Add 100 μL of Proteinase K solution in 4 mL of lysis buffer and mix well (*see* **Note 4.2-2**).
3. Add 200 μL of lysis buffer containing Proteinase K to the sampling tube and digest the tail sample at 56 °C for 120 min (see **Note 4.2-3**).

4. Inactivate the Proteinase K at 95 °C for 10 min and mix the solution by vortexing.
5. Prepare the reaction tubes. In addition to the samples, the following positive and negative controls are needed.
   (a) Human genomic DNA (positive control for hPVR).
   (b) Mouse (wild-type) genomic DNA (positive control for mPrr-2).
   (c) Lysis buffer (negative control).
   (d) At least three samples of $PVR^{+/+}$ genomic DNA ($PVR^{+/+}$ control), i.e., use genomic DNA from the original $PVR^{+/+}$ mice.
   (e) At least three samples of $PVR^{+/-}$ genomic DNA ($PVR^{+/-}$ control), i.e., use genomic DNA from the F1 mice.
6. Prepare the master mix without template DNA (*see* **Notes 4.2-4** and **5**).

×10 PCR buffer	2.0 μL
dNTP (2 mM each)	2.5 μL
$MgCl_2$ (25 mM)	2.0 μL
Primer PVR-4F (5 μM)	2.0 μL
Primer MPH-4R (5 μM)	2.0 μL
Taq DNA polymerase (5 units/μL)	0.1 μL
H_2O	12.4 μL (per tube)

7. Add 2 μL of template DNA to the reaction mixture.
8. Amplify the DNA using the following parameters.

Step 1	94 °C for 10 min	
Step 2	94 °C for 30 s	
Step 3	55 °C for 15 s	
Step 4	72 °C for 15 s	(Go back to Step 2 and repeat for 35 cycles)

9. Prepare the *Msp*I enzyme mixture.

*Msp*I (6–20 units/μL)	0.5 μL
× 10 M buffer	0.5 μL
H_2O	4 μL (per tube)

10. Add the enzyme mixture to each tube, mix by pipetting, and incubate at 37 °C for 2 h.
11. Load 5–10 μL of the sample onto a 4 % agarose (3 % NuSieve agarose + 1 % agarose) gel (*see* **Note 4.2-6**).

12. The length of the hPVR fragment is 109 bp. The mouse PRR-2 fragment contains an *Msp*I site and the fragment is cleaved into two fragments: 87 and 22 bp. Compare the intensity of the 109 bp human PVR fragment with that of the 87 bp mPRR-2 fragment (the 22 bp fragment might not be visible) by referring to the $PVR^{+/+}$ and $PVR^{+/-}$ controls. The intensity of the PVR fragment is higher than that of the mouse Prr-2 fragment in the $PVR^{+/+}$ control, whereas the intensities of the two fragments are almost the same in $PVR^{+/-}$ control (Fig. 3a) (*see* **Note 4.2–7** and **8**).
13. Prepare the reaction tubes to genotype the *Ifnar1* locus. In addition to the samples, the following positive and negative controls are required.
    (a) Mouse (wild-type) genomic DNA (positive control for $Ifnar1^{+/+}$).
    (b) Mouse (Ifnar1 KO) genomic DNA (positive control for $Ifnar1^{-/-}$).
    (c) Lysis buffer (negative control).
14. Prepare the master mix without template DNA.

×10 PCR buffer	2.0 μL
dNTP (2 mM each)	2.5 μL
$MgCl_2$ (25 mM)	2.0 μL
Primer IFNAR-51 (5 μM)	2.0 μL
Primer IFNAR-32 (5 μM)	2.0 μL
Primer neo-N3 (5 μM)	2.0 μL
Taq DNA Polymerase (5 units/μL)	0.1 μL
H_2O	10.4 μL (per tube)

15. Add 2 μL of template DNA to the reaction mixture.
16. Amplify the DNA using the following parameters.

Step 1	94 °C for 5 min	
Step 2	94 °C for 30 s	
Step 3	55 °C for 30 s	
Step 4	72 °C for 60 s	(Go back to Step 2 and repeat for 35 cycles)

17. Load 5–10 μL of the sample onto a 2 % agarose gel.
18. The wild-type and $Ifnar1^{-/-}$ alleles produce bands of 178 bp and 465 bp, respectively. Both bands are detected in the $Ifnar1^{+/-}$ genotype (Fig. 3b).
19. Select males and females with the $PVR^{+/+}$, $Ifnar1^{-/-}$ genotype.

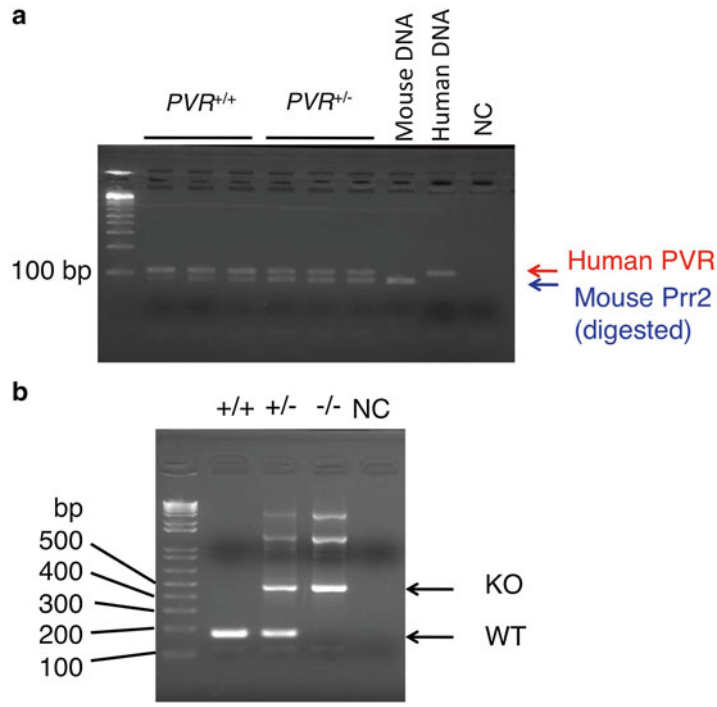

Fig. 3 Determination of the *PVR* and *Ifnar1* genotypes. (**a**) The PVR fragment and the Prr2 fragment (digested with *MspI*) run as bands of 109 bp and 87 bp, respectively. Note that the relative intensity of the PVR fragment to Prr2 fragment is higher in the $PVR^{+/+}$ genotype but not in the $PVR^{+/-}$ genotype. (**b**) The $Ifnar1^{+/+}$, $Ifnar1^{+/-}$, and $Ifnar1^{-/-}$ genotypes yield 178 bp, 178 + 465 bp, and 465 bp fragments, respectively

3.3 Detection of Poliovirus Antigens

Basically, immunohistochemical staining (IHC) and an indirect immunoperoxidase technique are used to detect virus antigens on sections (3 μm thick) of formalin-fixed, paraffin-embedded tissues. At present, polymer-based immunohistochemical systems are standard. These systems are based on a horseradish peroxidase (HRP)-labeled polymer, which is conjugated to secondary antibodies [44]. Commercial kits are available from many companies (*see* **Note 4.3-10**).

1. Inoculate the mice with poliovirus and maintain the infected mice in isolator racks until the day of sampling.
2. Prepare separate syringes filled with 15 mL of PBS and 25 mL of 4 % PFA. Connect the butterfly catheter and two syringes via a three-way stopcock. Purge any air bubbles from the line.
3. Euthanize the infected mice via the inhalation of excess isoflurane.
4. Immediately after breathing stops, open the abdomen and the thoracic cavity, taking care not to cut the major vessels.

5. Cut the right atrium to make a drain using small scissors. Insert the butterfly needle into the left ventricle at the apex toward the aorta and perfuse with 15 mL of PBS first (until the fluid is clear of blood).
6. Adjust the stopcock to perfuse 25 mL of 4 % PFA (*see* **Notes 4.3-1** and **2**).
7. Remove the organs and place them in 4 % PFA solution. Immerse the organs overnight (at least) (*see* **Note 4.3-3**).
8. Remove fat by immersing the organs in 50 % ethanol (three changes, each immersion lasting for 3 days).
9. Decalcify the bones by rocking in the decalcification solution. Change the solution every 3 days (three changes in all) (*see* **Note 4.3-4**).
10. Cut the fixed tissues into appropriate sizes and place in the embedding cassettes (*see* **Note 4.3-5**).
11. Dehydrate the tissues before paraffin embedding by immersing the cassette in the following sequence:
    - 70 % ethanol, two changes, 1 h each, at room temperature (r.t.) with rocking.
    - 80 % ethanol, two changes, 1 h each, at r.t. with rocking.
    - 95 % ethanol, two changes, 1 h each, at r.t. with rocking.
    - 100 % ethanol, three changes, 1 h each, at r.t. with rocking.
    - Xylene, three changes, 1 h each.
    - Paraffin wax, two changes, 1.5 h each at 56 °C.
12. Embed the tissues in paraffin blocks on the heating block.
13. Cool the blocks to allow the paraffin to solidify.
14. Cut off any excess paraffin from the embedded sample.
15. Mount the sample sections on a microtome.
16. Cut 3 μm slices using the microtome.
17. Place the paraffin slices in water at 40–45 °C.
18. Remove the paraffin sections from the water with glass slides and use a brush to position the sections.
19. Dry the sections overnight at 37 °C.
20. Deparaffinize the sections.
    - Wash the slides with three changes of xylene for 5 min each.
21. Rehydrate the sample sections.
    - Wash the slides with three changes of 100 % ethanol for 3 min each.
    - Wash the slides in 95 and 80 % ethanol for 3 min each.

22. Rinse the slides in running (distilled) water for 5 min.
23. Antigen retrieval reaction (*see* **Note 4.3-6**).
    - Heat the slides in retrieval solution (i.e., 10 mM sodium citrate buffer, pH 6.0, at 121 °C for 10 min in an autoclave).
24. After cooling, immerse the glass slides in 3 % hydrogen peroxide in methanol for 30 min at r.t.
25. Wash the slides with three changes of PBS for 5 min each.
26. Block nonspecific binding sites with 10 % normal goat serum in PBS for 5 min at r.t.
27. After draining the blocking solution, add the primary antibody (i.e., rabbit anti-poliovirus serum) at an appropriate dilution (1:500–1:4000) and incubate overnight at 4 °C (*see* **Notes 4.3-7** and **8**).
28. Wash the slides with three changes of PBS for 5 min each.
29. Apply the labeled secondary antibody (i.e., goat anti-rabbit Ig conjugated with dextran polymer on HRP molecules) for 30 min at r.t.
30. Wash the slides with three changes of PBS for 5 min each.
31. React with the chromogen substrate for 5–10 min (i.e., DAB).
32. Stop the color reaction by washing in distilled water.
33. Immerse the slides in aqueous hematoxylin to counterstain.
34. Wash the slides in running (distilled) water for 5 min.
35. Dehydrate the slides using two or three changes of 80, 95, and 100 % ethanol for 1 min each (*see* **Note 4.3–9**).
36. Clear with xylene.
37. Mount the coverslips using mounting medium.
38. Examine the mounted sections and confirm the antigens under a microscope (*see* **Note 4.3–11**).

4 Notes

4.1 Mouse Neurovirulence Test of Poliovirus via Intracerebral Inoculation

1. The sensitivity of mice to poliovirus infection varies with age. In particular, mice aged <5 weeks are highly sensitive to poliovirus. Thus, stable results are obtained if 6- to 7-week-old mice are used. Mice older than 10 weeks are not recommended because the skull is harder and it is difficult to control the depth of the needle.
2. The log LD_{50} or PD_{50} values for virulent strains are about 2–3, whereas those of attenuated strains are >6.
3. If anesthesia apparatus is not available, anesthetize the mice via intraperitoneal injection of 100 mg/kg of ketamine and 2 mg/kg of xylazine in saline.

4. The thick part of the needle helps to prevent it from extending too deeply into the brain, but the injection must be performed as carefully as possible.
5. Mice must be excluded from the dataset if they die on the day of inoculation or on the next day. This is because they might die due to injuries sustained during the injection.
6. If virulent strains are inoculated, all mice showing clinical signs will die. However, some mice will survive with paralysis if attenuated strains are inoculated.

4.2 Genotyping Protocol for the PVR *Gene*

1. Ten-day-old mice are suitable for sampling. Younger mice are too small to mark the ears by cutting. Mice at 2 weeks-of-age move briskly and are difficult to handle. To avoid cross-contamination, the scissors and forceps should be wiped with absorbent cotton soaked with 70 % ethanol and the residual ethanol must be burned after each operation.
2. Avoid storing Proteinase K at low concentrations. The lysis buffer containing Proteinase K should be prepared immediately before use.
3. Ensure that the small piece of the tail in each tube is soaked in the lysis buffer.
4. The sequences of the primers PVR-4F and MHP-4R do not match perfectly with those of Prr-2 and PVR, respectively; however, they are sufficiently homologous for amplification. Under the conditions used, the primers amplify both genes with almost equal efficiency.
5. This is a competitive PCR, and the theoretical ratio of the two genes does not change before and after amplification. The number of copies of the *PVR* gene in $PVR^{+/+}$ mice is over twice that in $PVR^{+/-}$ mice, whereas the number of copies of the *PRR-2* gene is the same.
6. This assay is delicate. Prepare the gels and run the samples very carefully. It is better to use a wide comb. Use at least three control samples from $PVR^{+/+}$ and $PVR^{+/-}$ mice, and the samples should be loaded on the same gel.
7. After obtaining the male and female $PVR^{+/+}$ candidates, cross them and check that all of the progeny have the same $PVR^{+/+}$ genotype. If all the samples are $PVR^{+/+}$, it can be concluded that they are homozygotes.
8. If the results are unclear, backcross the $PVR^{+/+}$ candidate with wild-type mice to confirm homozygosity.

4.3 Detection of Poliovirus Antigens

1. If perfusion is successful, the liver is bleached soon after perfusion with PBS and muscle contractions are observed soon after beginning perfusion with 4 % PFA. The mouse should be stiff when perfusion is finished.

2. If fluid drips from the mouse's nose, this indicates that the fluid pressure is too high. In this case, reduce the rate of perfusion.
3. During immersion fixation, the volume of 4 % PFA should be at least ten times the weight of the organs.
4. The decalcification step is optional. This step can be omitted if the organs can be excised from the bone.
5. Do not allow the tissues to dry at any time during the staining procedure.
6. Antigen retrieval is often performed with rehydrated sections by heat-mediated retrieval using EDTA buffer (pH 9.0) or citric acid buffer (pH 6.0) as the retrieval solution.
7. The optimal dilutions of antibody used to recognize the poliovirus antigen should be determined based on experiments with positive control tissue samples, such as tissue sections of formalin-fixed, paraffin-embedded cultures or poliovirus-infected animals.
8. The negative controls comprise samples from a non-infected animal or a sequential tissue section cut from each block and incubated with non-immune serum.
9. After reacting with the chromogen substrate, the rehydration and clearing steps should only be used if the chromogen substrate is insoluble in alcohol.
10. Polymer-based systems generally use a two-step IHC staining technique. The labeled polymer is an avidin- or biotin-free system, which avoids false-positive staining due to endogenous avidin-biotin activity. This system is extremely sensitive, and the optimal dilutions of the primary antibody are up to 20 times higher than those used by the traditional peroxidase anti-peroxidase (PAP) technique, as well as being several times higher than those used by the traditional avidin-biotin conjugate (ABC) or labeled avidin-biotin (LSAB) systems [44].
11. Poliovirus antigens can be detected successfully in CNS tissues when mice are fixed on the day of paralysis onset. However, detection becomes difficult at later times because of viral antigen clearance by the host. The detection limit is approximately 10^7 PFU/g. It is difficult to detect the antigens if the viral load is lower.

References

1. Hsiung GD, Black FL, Henderson JR (1964) Susceptibility of primates to viruses in relation to taxonomic classification. In: Buettner-Jaenusch J (ed) Evolutionary and genetic biology of primates, vol 2. Academic, New York, pp 1–23
2. Ida-Hosonuma M, Sasaki Y, Toyoda H, Nomoto A, Gotoh O, Yonekawa H, Koike S (2003) Host range of poliovirus is restricted to simians because of a rapid sequence change of the poliovirus receptor gene during evolution. Arch Virol 148(1):29–44

3. Armstrong C (1941) Cotton rats and white mice in poliomyelitis research. Am J Public Health Nations Health 31(3):228–232
4. Holland JJ (1961) Receptor affinities as major determinants of enterovirus tissue tropisms in humans. Virology 15:312–326
5. Holland JJ, Mc LL, Syverton JT (1959) Mammalian cell-virus relationship. III Poliovirus production by non-primate cells exposed to poliovirus ribonucleic acid. Proc Soc Exp Biol Med 100(4):843–845
6. Holland JJ, Mc LL, Syverton JT (1959) The mammalian cell-virus relationship. IV Infection of naturally insusceptible cells with enterovirus ribonucleic acid. J Exp Med 110(1):65–80
7. Bergelson JM (2010) Receptors. In: Roos RP, Ehrenfeld E, Domingo E (eds) The picornaviruses. ASM Press, Washington, DC, pp 73–86
8. Siddique T, McKinney R, Hung WY, Bartlett RJ, Bruns G, Mohandas TK, Ropers HH, Wilfert C, Roses AD (1988) The poliovirus sensitivity (PVS) gene is on chromosome 19q12 q13.2. Genomics 3(2):156–160
9. Mendelsohn C, Johnson B, Lionetti KA, Nobis P, Wimmer E, Racaniello VR (1986) Transformation of a human poliovirus receptor gene into mouse cells. Proc Natl Acad Sci U S A 83(20):7845–7849
10. Mendelsohn CL, Wimmer E, Racaniello VR (1989) Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell 56(5):855–865
11. Koike S, Horie H, Ise I, Okitsu A, Yoshida M, Iizuka N, Takeuchi K, Takegami T, Nomoto A (1990) The poliovirus receptor protein is produced both as membrane-bound and secreted forms. EMBO J 9(10):3217–3224
12. Koike S, Ise I, Nomoto A (1991) Functional domains of the poliovirus receptor. Proc Natl Acad Sci U S A 88(10):4104–4108
13. Selinka HC, Zibert A, Wimmer E (1991) Poliovirus can enter and infect mammalian cells by way of an intercellular adhesion molecule 1 pathway. Proc Natl Acad Sci U S A 88(9): 3598–3602
14. Ravens I, Seth S, Forster R, Bernhardt G (2003) Characterization and identification of Tage4 as the murine orthologue of human poliovirus receptor/CD155. Biochem Biophys Res Commun 312(4):1364–1371
15. Morrison ME, He YJ, Wien MW, Hogle JM, Racaniello VR (1994) Homolog-scanning mutagenesis reveals poliovirus receptor residues important for virus binding and replication. J Virol 68(4):2578–2588
16. Aoki J, Koike S, Ise I, Sato-Yoshida Y, Nomoto A (1994) Amino acid residues on human poliovirus receptor involved in interaction with poliovirus. J Biol Chem 269(11):8431–8438
17. Ren RB, Costantini F, Gorgacz EJ, Lee JJ, Racaniello VR (1990) Transgenic mice expressing a human poliovirus receptor: a new model for poliomyelitis. Cell 63(2):353–362
18. Koike S, Taya C, Kurata T, Abe S, Ise I, Yonekawa H, Nomoto A (1991) Transgenic mice susceptible to poliovirus. Proc Natl Acad Sci U S A 88(3):951–955
19. Sabin A, Boulger L (1973) History of Sabin attenuated poliovirus oral live vaccine strains. J Biol Stand 1:115–118
20. Nomoto A, Omata T, Toyoda H, Kuge S, Horie H, Kataoka Y, Genba Y, Nakano Y, Imura N (1982) Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. Proc Natl Acad Sci U S A 79(19): 5793–5797
21. Omata T, Kohara M, Kuge S, Komatsu T, Abe S, Semler BL, Kameda A, Itoh H, Arita M, Wimmer E et al (1986) Genetic analysis of the attenuation phenotype of poliovirus type 1. J Virol 58(2):348–358
22. Kawamura N, Kohara M, Abe S, Komatsu T, Tago K, Arita M, Nomoto A (1989) Determinants in the 5′ noncoding region of poliovirus Sabin 1 RNA that influence the attenuation phenotype. J Virol 63(3):1302–1309
23. Macadam AJ, Pollard SR, Ferguson G, Skuce R, Wood D, Almond JW, Minor PD (1993) Genetic basis of attenuation of the Sabin type 2 vaccine strain of poliovirus in primates. Virology 192(1):18–26
24. Westrop GD, Wareham KA, Evans DM, Dunn G, Minor PD, Magrath DI, Taffs F, Marsden S, Skinner MA, Schild GC et al (1989) Genetic basis of attenuation of the Sabin type 3 oral poliovirus vaccine. J Virol 63(3):1338–1344
25. Haller AA, Stewart SR, Semler BL (1996) Attenuation stem-loop lesions in the 5′ noncoding region of poliovirus RNA: neuronal cell-specific translation defects. J Virol 70(3): 1467–1474
26. Svitkin YV, Cammack N, Minor PD, Almond JW (1990) Translation deficiency of the Sabin type 3 poliovirus genome: association with an attenuating mutation C472 U. Virology 175(1):103–109
27. Horie H, Koike S, Kurata T, Sato-Yoshida Y, Ise I, Ota Y, Abe S, Hioki K, Kato H, Taya C et al (1994) Transgenic mice carrying the human poliovirus receptor: new animal models for study of poliovirus neurovirulence. J Virol 68(2):681–688
28. Abe S, Ota Y, Doi Y, Nomoto A, Nomura T, Chumakov KM, Hashizume S (1995) Studies on neurovirulence in poliovirus-sensitive transgenic

mice and cynomolgus monkeys for the different temperature-sensitive viruses derived from the Sabin type 3 virus. Virology 210(1):160–166

29. Abe S, Ota Y, Koike S, Kurata T, Horie H, Nomura T, Hashizume S, Nomoto A (1995) Neurovirulence test for oral live poliovaccines using poliovirus-sensitive transgenic mice. Virology 206(2):1075–1083. doi:10.1006/viro.1995.1030
30. Dragunsky E, Nomura T, Karpinski K, Furesz J, Wood DJ, Pervikov Y, Abe S, Kurata T, Vanloocke O, Karganova G, Taffs R, Heath A, Ivshina A, Levenbook I (2003) Transgenic mice as an alternative to monkeys for neurovirulence testing of live oral poliovirus vaccine: validation by a WHO collaborative study. Bull World Health Organ 81(4):251–260
31. Koike S, Taya C, Aoki J, Matsuda Y, Ise I, Takeda H, Matsuzaki T, Amanuma H, Yonekawa H, Nomoto A (1994) Characterization of three different transgenic mouse lines that carry human poliovirus receptor gene—influence of the transgene expression on pathogenesis. Arch Virol 139(3-4):351–363
32. WHO (2012) Standard operating procedure neurovirulence test of types 1, 2 OR 3 live attenuated poliomyelitis vaccines (oral) in transgenic mice susceptible to poliovirus, Version 6. World Health Organization, Switzerland
33. Kew O, Morris-Glasgow V, Landaverde M, Burns C, Shaw J, Garib Z, Andre J, Blackman E, Freeman CJ, Jorba J, Sutter R, Tambini G, Venczel L, Pedreira C, Laender F, Shimizu H, Yoneyama T, Miyamura T, van Der Avoort H, Oberste MS, Kilpatrick D, Cochi S, Pallansch M, de Quadros C (2002) Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. Science 296(5566):356–359. doi:10.1126/science.1068284
34. Jegouic S, Joffret ML, Blanchard C, Riquet FB, Perret C, Pelletier I, Colbere-Garapin F, Rakoto-Andrianarivelo M, Delpeyroux F (2009) Recombination between polioviruses and co-circulating Coxsackie A viruses: role in the emergence of pathogenic vaccine-derived polioviruses. PLoS Pathog 5(5):e1000412
35. Rakoto-Andrianarivelo M, Guillot S, Iber J, Balanant J, Blondel B, Riquet F, Martin J, Kew O, Randriamanalina B, Razafinimpiasa L, Rousset D, Delpeyroux F (2007) Co-circulation and evolution of polioviruses and species C enteroviruses in a district of Madagascar. PLoS Pathog 3(12):e191. doi:10.1371/journal.ppat.0030191
36. Shimizu H, Thorley B, Paladin FJ, Brussen KA, Stambos V, Yuen L, Utama A, Tano Y, Arita M, Yoshida H, Yoneyama T, Benegas A, Roesel S, Pallansch M, Kew O, Miyamura T (2004) Circulation of type 1 vaccine-derived poliovirus in the Philippines in 2001. J Virol 78(24):13512–13521
37. Thorley B, Kelly H, Nishimura Y, Yoon YK, Brussen KA, Roberts J, Shimizu H (2009) Oral poliovirus vaccine type 3 from a patient with transverse myelitis is neurovirulent in a transgenic mouse model. J Clin Virol 44(4):268–271. doi:10.1016/j.jcv.2009.01.014
38. Ida-Hosonuma M, Iwasaki T, Yoshikawa T, Nagata N, Sato Y, Sata T, Yoneyama M, Fujita T, Taya C, Yonekawa H, Koike S (2005) The alpha/beta interferon response controls tissue tropism and pathogenicity of poliovirus. J Virol 79(7):4460–4469
39. Abe Y, Fujii K, Nagata N, Takeuchi O, Akira S, Oshiumi H, Matsumoto M, Seya T, Koike S (2012) The toll-like receptor 3-mediated antiviral response is important for protection against poliovirus infection in poliovirus receptor transgenic mice. J Virol 86(1):185–194. doi:10.1128/JVI.05245-11
40. Oshiumi H, Okamoto M, Fujii K, Kawanishi T, Matsumoto M, Koike S, Seya T (2011) The TLR3/TICAM-1 pathway is mandatory for innate immune responses to poliovirus infection. J Immunol 187(10):5320–5327. doi:10.4049/jimmunol.1101503
41. Kärber G (1931) Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch Exp Pathol Pharmakol 162(4):480–483. doi:10.1007/BF01863914
42. Reed LJ, Muench H (1938) A simple method of estimating fifty per cent endpoints. Am J Hyg 27(3):493–497
43. Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, Aguet M (1994) Functional role of type I and type II interferons in antiviral defense. Science 264(5167):1918–1921
44. Sabattini E, Bisgaard K, Ascani S, Poggi S, Piccioli M, Ceccarelli C, Pieri F, Fraternali-Orcioni G, Pileri SA (1998) The EnVision++ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate, CSA, LABC, and SABC techniques. J Clin Pathol 51(7):506–511