

Original Article

Establishment of a panel of in-house polyclonal antibodies for the diagnosis of enterovirus infections

Osamu Kotani,^{1,3} Naoko Iwata-Yoshikawa,¹ Tadaki Suzuki,¹ Yuko Sato,¹ Noriko Nakajima,¹ Satoshi Koike,⁴ Takuya Iwasaki,¹ Tetsutaro Sata,¹ Teruo Yamashita,⁵ Hiroko Minagawa,⁵ Fumihiro Taguchi,³ Hideki Hasegawa,¹ Hiroyuki Shimizu² and Noriyo Nagata¹

Departments of ¹Pathology and ²Virology II, National Institute of Infectious Diseases, ³Department of Virology and Viral Infections, Faculty of Veterinary Medicine, Nippon Veterinary and Life Science University, ⁴Neurovirology Project, Tokyo Metropolitan Institute of Medical Science and ⁵Department of Microbiology and Medical Zoology, Aichi Prefectural Institute of Public Health, Aichi, Japan

The aim of this study was to establish a reliable method of virus detection for the diagnosis of critical enterovirus infections such as acute infective encephalitis, encephalomyelitis and myocarditis. Because histopathological and immunohistochemical analyses of paraffin-embedded tissues play an important role in recognizing infectious agents in tissue samples, six in-house polyclonal antibodies raised against three representative enteroviruses using an indirect immunofluorescence assay and immunohistochemistry were examined. This panel of polyclonal antibodies recognized three serotypes of enterovirus. Two of the polyclonal antibodies were raised against denatured virus particles from enterovirus A71, one was raised against the recombinant VP1 protein of coxsackievirus B3, and the other for poliovirus type 1 were raised against denatured virus particles, the recombinant VP1 protein and peptide 2C. Western blot analysis revealed that each of these antibodies recognized the corresponding viral antigen and none cross-reacted with non-enteroviruses within the family *Picornaviridae*. However, all cross-reacted to some extent with the antigens derived from other serotypes of enterovirus. Indirect immunofluorescence assay and immunohistochemistry revealed that the virus capsid and non-structural proteins were localized in the cytoplasm of affected culture cells, and skeletal muscles and neurons in neonatal mice experimentally-infected with human enterovirus. The antibodies also

recognized antigens derived from recent clinical isolates of enterovirus A71, coxsackievirus B3 and poliovirus. In addition, immunohistochemistry revealed that representative antibodies tested showed the same recognition pattern according to each serotype. Thus, the panel of in-house anti-enterovirus polyclonal antibodies described herein will be an important tool for the screening and pathological diagnosis for enterovirus infections, and may be useful for the classification of different enterovirus serotypes, including coxsackieviruses A and B, echoviruses, enterovirus A71 and poliovirus.

Key words: enterovirus, immunohistochemistry, paraffin-embedded tissue, polyclonal antibody, viral encephalitis.

INTRODUCTION

Enterovirus (EV), one of the genres of the family *Picornaviridae*, including coxsackievirus A and B (CVA and CVB), echovirus (Echo), enterovirus A71 (EV71), and poliovirus (PV) cause a wide spectrum of human disease, such as hand-foot-and-mouth disease (HFMD), herpangina, pneumonia, diabetes mellitus and aseptic meningitis. EV71 and CVB infections possibly cause fatal severe diseases, such as meningoencephalitis and myocarditis.^{1–10} It has been unclear how the patients developed severe diseases from a virus that causes HFMD and/or mild meningitis.

EV is a positive-sense, single-stranded RNA virus. The viral capsid comprises four proteins: VP1, VP2, VP3 and VP4. The epitopes on VP1, VP2 and VP3 are recognized by neutralizing antibodies.^{11–13} In particular, VP1 bears the greatest number of neutralizing epitopes.^{14–16} Serologically,

Correspondence: Noriyo Nagata, DVM, PhD, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. Email: nnagata@niid.go.jp

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EV is categorized into 66 or more serotypes on the basis of neutralization tests using prototype viruses. Neutralizing antibodies have also been used to identify EV in neonatal mice and tissue cultures. The ninth report by the International Committee on Taxonomy of Viruses in 2013 reclassified the genus *Enterovirus* into 12 species (*EV-A*, *-B*, *-C*, *-D*, *-E*, *-F*, *-G*, *-H*, *-J* and *Rhinovirus A*, *B*, *C*) according to their biological and genetic properties.¹⁷

Detection of pathogens is necessary for accurate pathological diagnoses and to identify the relationship between a pathogen and an illness.¹⁸ Immunohistochemistry (IHC) and *in situ* hybridization (ISH) are used to detect viral antigens and its genomes on tissue sections, respectively.^{1,19–23} Nowadays, reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real time PCR are also used to detect viral genomes using nucleic acids extracted from fresh tissues and/or paraffin-embedded tissue sections.^{24–28} However, RNA and DNA genomes are easily modified during formalin fixation.²⁹ After more than 3 weeks of formalin fixation, the sensitivity for detecting viral RNA in paraffin-embedded tissues falls by approximately 100 times compared with that in fresh tissues.³⁰ Thus, detecting viral antigens by IHC is most useful for screening and diagnostic procedures. Paraffin-embedded tissue sections from virus-infected neonatal mice or cultured cells can be used as positive controls when detecting viral antigens or genomes by IHC or ISH.^{24,31,32} Some researchers used cultured cells as positive controls for IHC and ISH,^{21,24,28,31} whereas others used tissues from virus-infected neonatal mice as positive controls.^{20,22}

We previously developed, three in-house anti-EV polyclonal antibodies, which were raised denatured virus particles derived from EV71 and PV, for diagnosis and pathological research into disease pathogenesis in experimental animals.^{23,33–37} Here, we generated another three anti-EV polyclonal antibodies specific for the CVB3 VP1 protein, PV VP1 protein and the PV1 2C non-structural protein. All six anti-EV polyclonal antibodies were examined in terms of their specificity for, and cross-reactivity with, different enterovirus serotypes, species and genera. We found that the polyclonal antibodies were able to recognize several EV serotypes, including CVA, CVB, Echo, EV71 and PV.

MATERIALS AND METHODS

Viruses and cells

All EV examined in this study are listed in Table 1 with clinical diagnoses or symptoms, genotype, accession number, cells using the assays, and infectivity to neonatal mice. The predominant serotypes of EV isolations from the cases of aseptic meningitis in Japan during a 5-year period

(2007–2011) were selected for this study.³⁸ Rhesus monkey kidney epithelial (LLC-MK₂) cells and rhabdomyosarcoma (RD) cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). The cells and prototype viruses were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The polyclonal antibodies were tested against EV71, PV and CVB3 isolates to examine their utility for detecting clinical isolates in cell culture. The isolates were classified using a neutralization assay with virus-specific antisera, or by examining the capsid region sequences. The EV71 strains were isolated between 1997 and 2010^{39,40} and the CVB3 strains were isolated between 2011 and 2012 in Japan. The PV1 and PV2 strains were isolated from patients with acute flaccid paralysis (associated with vaccine-derived poliovirus) in the Philippines in 2001 and Nigeria in 2005, respectively.^{41,42} Human parechovirus (HPeV), Saffold virus (SAFV) and encephalomyocarditis virus (EMCV) were used to assess the polyclonal antibodies existing in any cross-reactivity against other picornaviruses. The JPN08-404 strain of SAFV was isolated in 2008 at the Public Health Institute of Kochi Prefecture in Japan and was kindly supplied by Dr. T. Hosomi.⁴³

Virus-infected cells were disrupted by two or three freeze-thaw cycles to prepare virus solutions, and the 50% cell culture infective doses (CCID₅₀) which were calculated by the Behrens–Kärber method on the basis of the cytopathic effects (CPE) observed in LLC-MK₂ cells monolayers incubated with varying dilutions were determined before animal inoculations.

Polyclonal antibodies

Six polyclonal antibodies (all prepared in-house) were used for the study, three of which were raised against the denatured virus particles from C7/Osaka/Japan 97 isolate of EV71 (anti-EV71-C7; genogroup B according to phylogenetic analysis of the VP1 region), the 1095/Japan 97 isolate of EV71 (anti-EV71-1095; genogroup C) and the Mahoney strain of PV1 (anti-PV1-OM1).³⁴ These three antibodies were produced by immunizing New Zealand White rabbits with viral particles that had been denatured in 0.5% sodium dodecyl sulfate (SDS)/0.1 mol/L phosphate buffered saline (PBS) at 80°C for 10 min as previously described.^{34,44} Two polyclonal antibodies against the CVB3 and PV1-VP1 protein (anti-CVB3-VP1 and anti-PV1-VP1) were prepared as follows. The VP1 region of CVB3⁴⁵ and PV1⁴⁶ cDNA was amplified by PCR and the fragment was cloned into the BamHI and EcoRI sites of the pGEX-3X vector (GE Healthcare Life Sciences, Little Chalfont, UK), following the procedure recommended by the manufacturer. The vector was then used to transform *Escherichia coli* strain BL21 (Takara Bio, Shiga, Japan). Expression of the GST-CVB3 VP1 and the GST-PV1 VP1 fusion proteins

Table 1 List of the subjected enteroviruses in the study

Species	Serotypes	Strains	Illness in Person or source	Genotype	Accession number	Infected cells		Infectivity to neonatal mouse			
						WB	IHC				
<i>Enterovirus A (EV-A)</i>	EV71	BrCr-A/39C-1tr	Poliomyelitis	A	U22521	NE	NE	RD	NE		
		SK-EV006/Malaysia 97	Meningitis	B3	AB469182.1	RD	RD	NE	NE	+	
		C7/Osaka/Japan 97	Encephalitis	B4	AB550336	NE	NE	RD	RD	NE	
		1095/Japan 97	HFMD	C2	AB059817	NE	NE	RD	RD	NE	
		A211/10	Fever	C2	AB935178	NE	NE	RD	RD	NE	
		75/Yamagata/Japan 97	HFMD	C4	AB177813	NE	NE	RD	RD	NE	
		Olson	Meningitis		AY421761.1	RD	RD	NE	NE	+	
		Shimane/RD1	Sewage		NE	RD	RD	NE	NE	+	
		Gdula	Meningitis		AY421764.1	RD	RD	NE	NE	+	
		Kowalik	Meningitis		AY421767.1	RD	RD	NE	NE	+	
	<i>Enterovirus B (EV-B)</i>	CVA3 CVA4 CVA6 CVA10 CVA16 CVB3	G-10	None		U05876.1	RD	RD	NE	NE	+
			Nancy	febrile illness		JX312064.1	RD	RD/LLC-MK ₂	NE	NE	+
			A10004/12	Fever		AB935177	NE	NE	RD	RD	NE
<i>Enterovirus C (EV-C)</i>	PV1 PV2 PV2 PV2 PV3	A201/11	Herpangina		AB934931	NE	NE	RD	RD	NE	
		Bozek	Meningitis		AJ295200	RD	LLC-MK ₂	NE	NE	+	
		pMP1.23	Pleurodynia		AY186746.1	RD	LLC-MK ₂	NE	NE	+	
		Ohio-1	Summer gripe		AF085363	RD	LLC-MK ₂	NE	NE	+	
		J.V.B. Benschoten	Chest and abdominal illness		X05690.1	RD	LLC-MK ₂	NE	NE	+	
		Faulkner	Mild paralytic disease with atrophy		AF114383.1	RD	LLC-MK ₂	NE	NE	+	
		D'Amori	Meningitis		AY302558.1	RD	RD	NE	NE	-	
		Hill	None		X84981.1	RD	RD	NE	NE	-	
		Gregory	None		X80059.1	RD	RD	NE	NE	-	
		JV-4	Diarrhea		AY302549.1	RD	RD	NE	NE	-	
		Bastianni	Meningitis		AF162711.1	RD	RD	NE	NE	-	
		Mahoney	Fatal paralytic poliomyelitis		NC_002058.3	RD	RD	NE	NE	-	
		Sabin1	Attenuated		V01150.1	NE	NE	RD	RD	NE	
<i>Enterovirus C (EV-C)</i>	PV1 PV2 PV2 PV2 PV3	PJ161 (Luzon-01-1)	AFP/ VDPV derived Sabin 1		AB180071	NE	NE	RD	RD	NE	
		PJ154 (Mindanao-01-1)	AFP/ VDPV derived Sabin 1		AB180070	NE	NE	RD	RD	NE	
		Lansing	Fatal paralytic poliomyelitis		M12197.1	RD	RD	NE	NE	NE	
		11196 (KTS09-02)	AFP/ VDPV derived Sabin 2		JX275166	NE	NE	RD	RD	NE	
		11198 (BOS07-02)	AFP/ VDPV derived Sabin 2		JX275052	NE	NE	RD	RD	NE	
		Leon	Fatal paralytic poliomyelitis		K01392	RD	RD	NE	NE	NE	

AFP, acute flaccid paralysis; BrCr-A/39C-1tr, temperature-resistant infectious clone strain; HFMD, hand-foot-and-mouth disease; IHC, immunohistochemistry; Indirect IFA, indirect immunofluorescence assay; LLC-MK₂, Rhesus monkey kidney epithelial cells; NE, not examined; RD, rhabdomyosarcoma cell; VDPV, vaccine-derived polioviruses; WB, Western blotting.

were induced by isopropyl-D-1-thiogalactopyranoside. The cell pellets were sonicated and the inclusion bodies containing the fusion protein were collected. The fusion proteins were purified by SDS-PAGE and used to immunize New Zealand White rabbits as previously described.⁴⁴ The antibody against PV1 2C (anti-PV1 2C) was generated as follows: the antibody was raised against peptide 2C within PV1, which comprises 20 amino acids and is well conserved among EV. Anti-PV1 2C was expected to detect many other EV. The following EV protein sequences were extracted from the GenBank database: EV-A: CVA3, AAR38841.1; CVA4, AAR38842.1; CVA6, AAR38844.1; CVA10, AAR38847.1; CVA16, AAA50478.1; and EV71, BAG82821.1; EV-B: CVA9, BAA00518.1; CVB1, AAO84299.1; CVB2, AAD19874.1; CVB3, AFS18536.1; CVB4, CAA29172.1; CVB5, AAF21971.1; Echo6, AAQ73095.1; Echo9, CAA59341.1; Echo11, CAA56365.1; Echo25, AY302549.1; and Echo30, AAD45119.1; EV-C: PV1, NP_041277.1; PV2, AAA46912.1; and PV3, AAA46914.1. The amino acid sequence of peptide 2C was as follows: CQMVSTVEFIPPMASLEEKG. The sequence showed high homology compared with other EV 2C peptides (*EV-A*: 95%, *EV-B*: 80%, *EV-C*: 100% of homology). This sequence was then submitted to the antibody production service at Sigma-Genosys (Sigma-Aldrich, St. Louis, MO, USA). A commercial mouse monoclonal anti-EV antibody (Clone 5-D8/1; Dako Denmark A/S, Glostrup, Denmark) was used as the positive control for immunofluorescence assay (IFA), Western blotting (WB) and IHC staining. This antibody reacts with the EV serotypes-specific epitopes of the capsid protein VP1.⁴⁷ Normal rabbit serum (Dako Denmark A/S) was used as a negative control for IFA, WB and IHC.

Western blotting

Cytopathic effects were observed in more than 80% of picornavirus-infected RD cells at 24 or 48 h post-infection. Infected cells were lysed in radio-immunoprecipitation assay buffer and cell debris was removed by centrifugation. Lysates from cells infected with infected with HPeV, SAFV or EMCV were used as negative controls. Picornavirus proteins were separated in NuPAGE 12% Bis-Tris Precast Gels (Novex, Life Technologies Corporation, Carlsbad, CA, USA) and then electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). The PVDF membrane was blocked with PVDF Blocking Reagent for Can Get Signal (Toyobo Co. Ltd, Osaka, Japan) at 4°C for overnight. The membrane was then incubated for 1 h with the appropriate primary antibody diluted 1:5000 in Can Get Signal Immunoreaction Enhancer Solution (Toyobo). After washing three times (for 15 min each) with 0.1% Tween 20 in Tris buffer solution, the membranes were incubated with

the appropriate horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, UK) for 1 h. Signals were developed using the Immobilon western chemiluminescent reagent (Merck Millipore) and detected using the ImageQuant LAS4000 digital imaging system (GE Healthcare UK Ltd., UK).

Immunofluorescence assay

LLC-MK₂ or RD cells were seeded in a Nunc 16 well chamber slide (Thermo Fisher Scientific Inc., Waltham, MA, USA) and cultured in MEM supplemented with 2% FBS for 1 day. The cells were inoculated with each picornavirus at a multiplicity of infection > 0.1 and incubated at 37°C for 12 h, 1 day, or 2 days. When CPE were recognized in less than 25% of the cells, the infected cell monolayer was fixed with 4% paraformaldehyde/4% sucrose in PBS at room temperature (RT) for 15 min followed by methanol/acetone at -30°C for 1 min. After three washes with 0.1% Triton X-100 in PBS, the cells were incubated for 30 min with PBS containing 10% FBS followed by an overnight incubation at 4°C with each anti-picornavirus antibody. After three washes with 0.1% Triton X-100 in PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L) (Molecular Probes, Life Technologies Corp., Carlsbad, CA, USA) antibodies for 30 min at RT. Cells were counterstained with *SlowFade* Gold antifade reagent with DAPI (Invitrogen, Life Technologies Corp.). Viral antigens (green) and cell nuclei (blue) were visualized using a laser scanning confocal microscope (FV1000-D; Olympus, Tokyo, Japan).

Experimental infection of mice and preparation of paraffin-embedded tissue sections

Pregnant ddY mice were purchased from Japan SLC (Shizuoka, Japan). Newborn mice were inoculated intracerebrally (i.c.) with 10 µL of virus solution. Mice were clinically observed and sacrificed during early infection or onset of serious neurological manifestation day ($n = 3$ or 4 on each of these days). Phosphate-buffered formalin (10%) was perfused directly into the heart while the mice were sacrificed with an excess dose of anesthesia with isoflurane. The tissues were then immersed in formalin solution overnight. The whole head, including the brain, was cut into sagittal sections, which were immersed in the formalin solution for a few days. After fixation, the sections were dehydrated in ethanol (30% to 50% solutions) and decalcified in buffered EDTA 2Na solution (Dojindo, Kumamoto, Japan). The samples were then embedded in paraffin and stained with HE. All animal experiments, including rabbit immunizations, were approved by the

Committee on Experimental Animals, National Institute of Infectious Diseases, Japan and all experimental animals were handled according to the guidelines set out by this Committee.

Preparation of EV-infected cell blocks

RD cells were infected with isolate strains of EV71 (four isolates), PV (four isolates and vaccine strain), and CVB3 (two isolates) and with prototype strains of Echo (6, 9, 11, 25 and 30) for 1 day. The cells were fixed with 10% phosphate-buffered formalin overnight, then embedded in iPGell (Geno Staff, Tokyo, Japan) followed by paraffin according to the manufacturer's instructions.

Immunohistochemical detection of viral antigens

A polymer-based detection system was used for immunohistochemical analysis. For antigen retrieval, deparaffinized sections were placed in retrieval solution (pH 6) (Nichirei Biosciences, Inc., Tokyo, Japan) and heated to 121°C for 10 min in an autoclave. After washing with PBS, the sections were treated for 30 min with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. After further washing, the sections were incubated in 10% normal goat serum (Dako Denmark A/S) at RT for 5 min, followed by an overnight incubation at 4°C with the primary anti-picornavirus polyclonal antibodies. After further washing in PBS, the sections were incubated with Nichirei-Histofine Simple Stain Mouse MAX PO (R) (Nichirei Biosciences) according to the manufacturer's protocol. Peroxidase activity was detected with 3,3'-diaminobenzidine (Sigma-Aldrich) and the sections were counterstained with hematoxylin. When a specific background was seen, acetone powder prepared from normal neonatal and adult mouse spleen, liver and brain tissues were used for blocking the spurious activities. The powder (1 mg) was added to each 1 mL of polyclonal sera and incubated for 4°C overnight. After centrifugation at 10 000 × g for 10 min, the supernatant was used for IHC.

Sequence analysis of viral genes

The EV VP1 gene sequences of EV71 and CVB3 isolates (A211/10, A10004/12 and A201/11) were amplified using the consensus-degenerate hybrid oligonucleotide primer (CODEHOP) VP1 reverse-transcription semi-nested PCR method and primers described by Nix *et al.*⁴⁸ Viral RNA was extracted from virus-infected cell culture using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). cDNA was generated using four different primers (primers AN32-35) and SuperScript II RNaseH-Reverse Transcriptase (Invitrogen, Life Technologies Corporation). The cDNA was then used in the first-round PCR with primers SO222

and SO224. The PCR product was then amplified in a second-round nested PCR using primers AN88 and AN89. The amplified DNA was sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems, Life Technologies Corp.). The VP1 nucleotide sequences were obtained from the National Institute for Public Health and the Environment (Bilthoven, The Netherlands) database using the EV Genotyping Tool (Version 1.0) to identify the EV serotypes with the highest sequence homology.⁴⁹ The nucleotide sequences of three EV isolates were aligned using Sequencher (ver. 4.10.1, Gene Codes Corporation, Ann Arbor, MI, USA). All nucleotide sequences analyzed in this study have been submitted to the DNA Data Bank of Japan (DDBJ) (Table 1).

RESULTS

Reactivity of the polyclonal antibodies with the enterovirus capsid and non-capsid proteins

The reactivity of antisera raised in rabbits was evaluated by WB against picornavirus proteins (derived from EV or non-EV-infected cell lysates) (Fig. 1). All of the in-house polyclonal antibodies were specific for their corresponding EV antigens, although they did show some cross-reactivity with other EV antigens. However, none of the polyclonal antibodies recognized non-EV-derived antigens, including HPeV, SAFV or EMCV. WB revealed that the anti-denatured picornavirus particle antibodies (anti-EV71-C7 and -1095 and anti-PV1-OM1) recognized mainly VP1 (approximately 33–34 kDa) from the corresponding viral strains, although again they did show some background staining (Fig. 1A and C; upper panels). The anti-EV71-C7 antibody cross-reacted with PV3 and the anti-EV71-1095 antibody showed broad cross-reactivity with CVA, CVB and PV. The anti-CVB3-VP1 antibody cross-reacted with VP1 (34 kDa) from *EV-B* serotypes, including CVB and Echos (Fig. 1B). The anti-PV1-VP1 antibody was specific for the VP1 protein of PV1, 2 and 3, and some *EV-B* serotypes (CVA9, CVB2 and Echo30) (Fig. 1C; middle panels). The anti-PV1 2C antibody recognized the PV2C antigen (37 kDa) and the 2C protein of all HEV serotypes (Fig. 1C; lower panels). A commercial anti-EV monoclonal antibody (clone 5-D8/1) detected the VP1 proteins of CVB, Echos 6 and 9, and PV1 (Fig. 1D).

Determining the specificity and cross-reactivity of the anti-EV in-house polyclonal antibodies using IFA

IFA was performed to examine both the specificity and cross-reactivity of the six in-house polyclonal antibodies for EV. First, we confirmed that all cultured cells infected

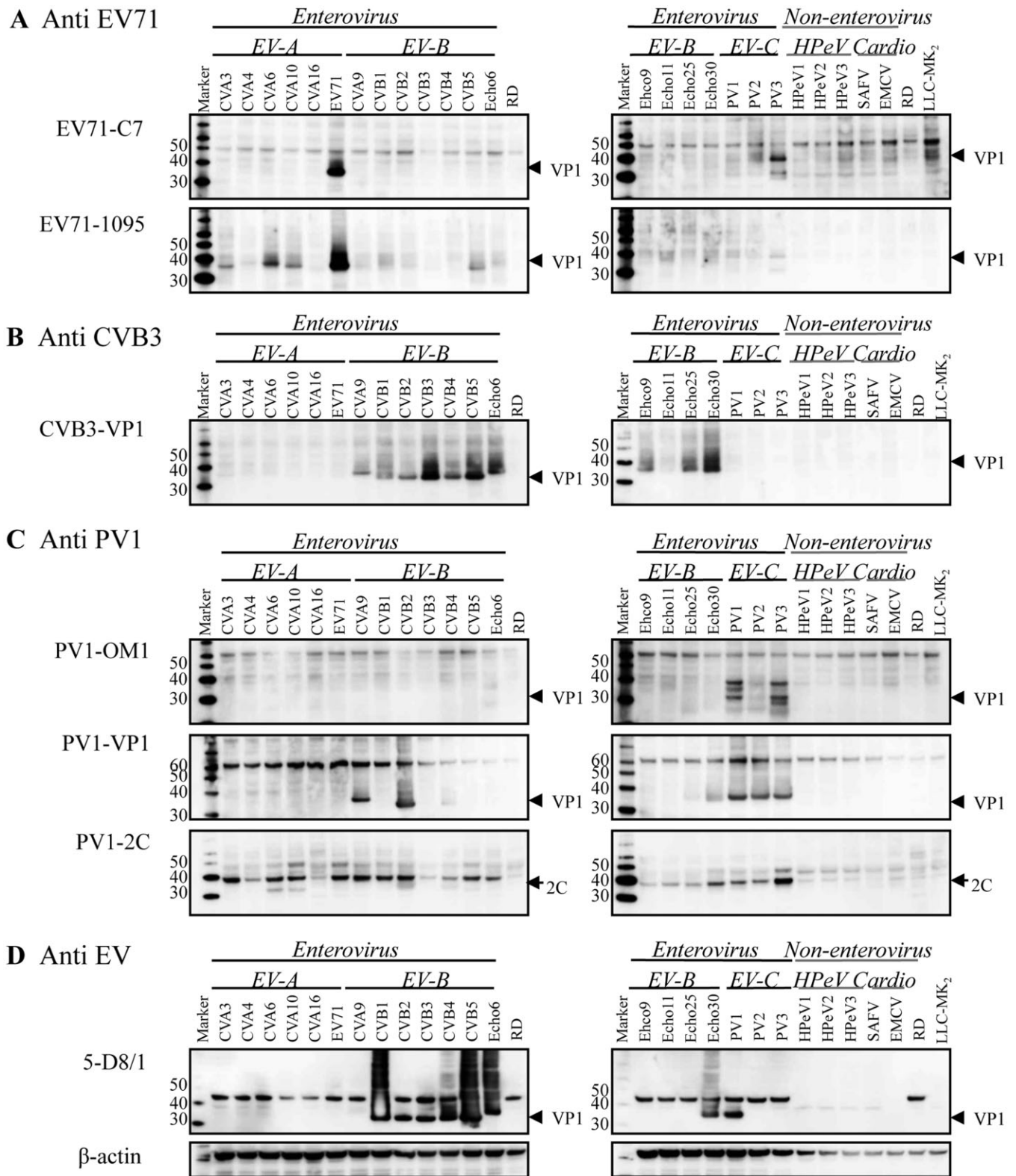


Fig. 1 Western blot analysis of viral proteins in lysates from rhabdomyosarcoma (RD) cells or Rhesus monkey kidney epithelial (LLC-MK₂) cells infected with human enteroviruses (EV). Blots were probed with the in-house anti-EV antibodies followed by a horseradish peroxidase-conjugated anti-rabbit immunoglobulin secondary antibody. β -actin was used as the control. Capsid proteins (VP1) derived from each EV were detected using rabbit antisera against EV71 (A, anti-EV71-C7 and anti-EV71-1095), an antibody against CVB3 (B, anti-CVB3-VP1), and antibodies against PV1 (C, anti-PV1-OM1 and anti-PV1-VP1). A commercial anti-EV antibody, 5-D8/1, (anti-EV) and anti- β -actin antibody were used as positive and internal controls, respectively (D).

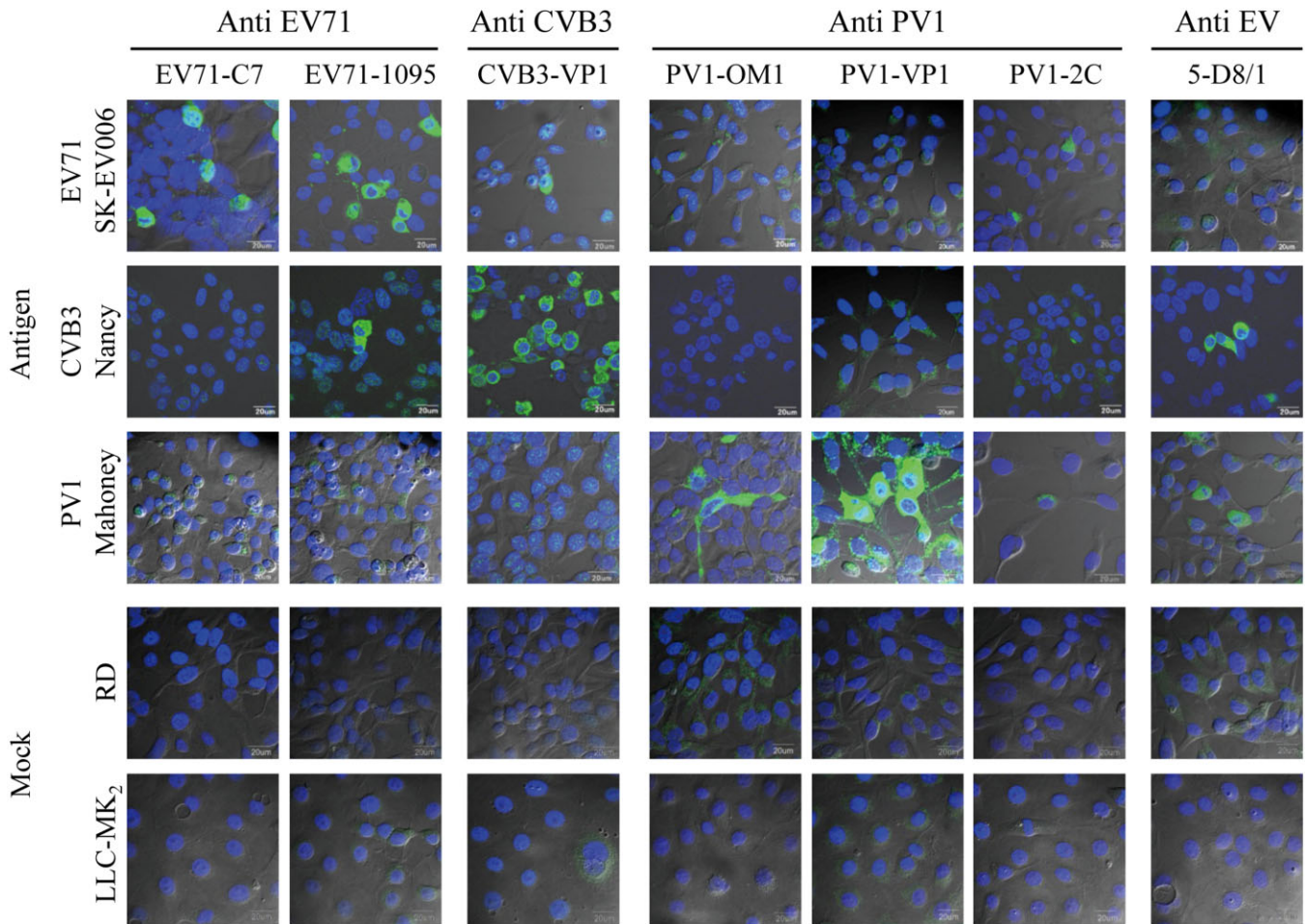


Fig. 2 Immunofluorescence assay using the six in-house anti-enterovirus (EV) antibodies. Paraformaldehyde-methanol/acetone-treated rhabdomyosarcoma (RD) cells infected with enterovirus A71 (EV71, SK-EV006/Malaysia 97), coxsackievirus B3 (CVB3, Nancy), or poliovirus type 1 (PV1, Mahoney) were used for the assay. A commercial anti-EV antibody, 5-D8/1, was used as the positive control. Magnification, $\times 60$. Scale bar = 20 μm .

with EV and non-EV serotypes showed CPE, including cytolysis and degeneration. All six in-house polyclonal antibodies detected virus antigens originating from virus-infected cells that had been fixed with 4% paraformaldehyde, methanol-acetone, and then lysed in Triton X-100. Degenerating cells were positive for viral antigens by IFA (Fig. 2). The anti-EV71-C7 and -1095, the anti-PV1-OM1, the anti-CVB3 and the anti-PV1-VP1 antibodies recognized the capsid antigen in the cell cytoplasm. The PV1 2C antigen was positive in perinuclear regions. The anti-EV71-C7 and anti-EV71-1095 antibodies cross-reacted with CVA, CVB and PV (Table 2). The anti-CVB3-VP1 antibody reacted with CVB antigens but also cross-reacted with CVA6 and some Echo strains, but not with PV. Both the anti-PV-VP1 and anti-PV1-OM1 antibodies recognized all PV serotypes and cross-reacted with a few EV-B serotypes. However, the anti-PV1 2C antibody recognized all three PV serotypes and showed broad cross-reactivity with CVA, EV71, CVB and Echo. None of the in-house

polyclonal antibodies recognized HPeV-, SAFV-, or EMCV-derived antigens and none reacted with non-infected cells.

Detection of viral capsid antigens and non-structural protein 2C in neonatal mice by IHC

Next, neonatal mice were infected with picornavirus and we examined the specificity of the polyclonal antibodies for viral antigens in paraffin-embedded mouse tissues by IHC. Previously described poliovirus-infected mouse tissue samples were also examined.^{35,36} Blocks of Echo-infected cells were used instead of tissue samples because Echo does not infect neonatal mice. Microscopic lesions, such as foci of degenerated cells, necrotic cells, and/or inflammation in skeletal muscles and nerve tissues were confirmed histopathologically by staining with HE (Fig. 3A, D and F). Almost all degenerated cells were positive for the viral capsid antigen (Fig. 3B, C, E and G-I). All of the in-house

Table 2 Summary of antibody specificity and cross-reactivity to enteroviruses in immunofluorescent assay and immunohistochemistry

Species	Serotypes	Indirect immunofluorescence assay of infected cells										Immunohistochemical staining of infected cells or animal tissues 1									
		Anti-EV71 EV71-C7	EV71-1095	Anti-CVB3 CVB3-VP1	Anti-PV1 PV1-OM1	PV1-VP1	PV1-2C	Anti-EV 5-D8/1	Anti-EV71 EV71-C7	EV71-1095	Anti-CVB3 CVB3-VP1	Anti-PV1 PV1-OM1	PV1-VP1	PV1-2C	Anti-EV 5-D8/1						
EV-A	CVA3	+	+	+	-	+	w	-	+	+	+	+	+	+	+						
	CVA4	+	-	+	-	+	w	-	+	+	+	+	+	+	+						
	CVA6	+	+	+	-	+	w	-	+	+	+	+	+	+	+						
	CVA10	+	-	+	-	+	w	-	+	+	+	+	+	+	+						
	CVA16	+	-	+	-	+	w	-	+	+	+	+	+	+	+						
	EV71	+	+	+	-	+	w	-	+	+	+	+	+	+	+						
	EV71-C7	+	+	+	-	+	w	-	+	+	+	+	+	+	+						
EV-B	CVA9	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	CVB1	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	CVB2	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	CVB3	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	CVB4	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	CVB5	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	Echo6	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	Echo9	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	Echo11	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	Echo25	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
EV-C	Echo30	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	PV1	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	PV2	+	+	+	+	+	w	-	+	+	+	+	+	+	+						
	PV3	+	+	+	+	+	w	-	+	+	+	+	+	+	+						

†EV-infected animal tissues were used. ‡Non-specific signals were detected; w, detected signals were weak; shaded columns were for homologous antibodies/antigens in the species.

polyclonal antibodies recognized their corresponding EV antigens, with some cross-reactivity with other EVs (Fig. 3 and Table 2). None of the in-house polyclonal antibodies recognized antigens derived from HPeV or SAFV. The commercial anti-EV monoclonal antibody showed strong but non-specific reactivity with mouse tissues.

In addition, IHC revealed that the anti-EV71-C7 antibody cross-reacted with CVA6, CVA16, CVB2, CVB5 and PV3 (Table 2). The anti-EV71-1095 antibody showed broad cross-reactivity with CVA-, CVB- and PV-derived antigens, whereas the anti-CVB3-VP1 antibody cross-reacted with CVA6 and EV-B, including CVB and Echo. Both anti-PV1-OM1 and anti-PV1-VP1 cross-reacted with all PV serotypes and with some EV-B. Anti-PV1-2C recognized all PV serotypes and EV-A, including CVA and EV71. Both the virus capsid antigen and the 2C viral antigen were detected in the cytoplasm of degenerated neurons in ICR mice infected with virulent PV2 (Lansing strain) (Fig. 3G-I).

In addition, we performed histopathological analysis of nerves of the brain and spinal cord, skeletal muscle of feet and heart, and mucosal epithelium of the oral cavity from neonatal ddY mice intracerebrally inoculated with EV prototype strains or clinical isolates (Table 3). IHC using in-house polyclonal antibodies identified viral antigens in pathological lesions in neonatal mice. CVA and EV71 viruses belong to the species *EV-A*. CVA9 (*EV-B*) appeared to mainly infect skeletal muscle and, in some cases, nerve cells and epithelial cells. However, CVB viruses, which belong to the *EV-B* species, infected a broad range of tissues, including neurons, skeletal muscle, mucosal epithelium, exocrine glands, liver and brown fat (data not shown).

Usefulness of the polyclonal antibodies for detecting recent clinical isolates in IHC

To further examine the utility of the polyclonal antibodies, we examined formalin-fixed paraffin-embedded cells that had been infected with 10 clinical isolates of EV71, CVB3 and PV obtained between 1997 and 2012 (Fig. 4). Six in-house polyclonal antibodies showed an identical recognition pattern for each serotype (Table 4). The amino acid sequences of the different virus isolates harbored some mutations. For example, compared with the amino acid sequence of the CVB3 Nancy strain, the sequence of the clinical isolate of CVB3 harbored eight amino acid changes (L638V, K650E, A654S, K655N, L622I, P664T, A668V and V680M) in the VP1 region spanning amino acids 611–657. On the other hand, the EV71 isolates (A211/10 and 75-Yamagata) each harbored two amino acid differences (A211/10, T623A, G710Q; 75-Yamagata, T623A, G710E) when compared with the VP1 sequence from EV71 C7-Osaka. The amino acid sequences of the PV isolates

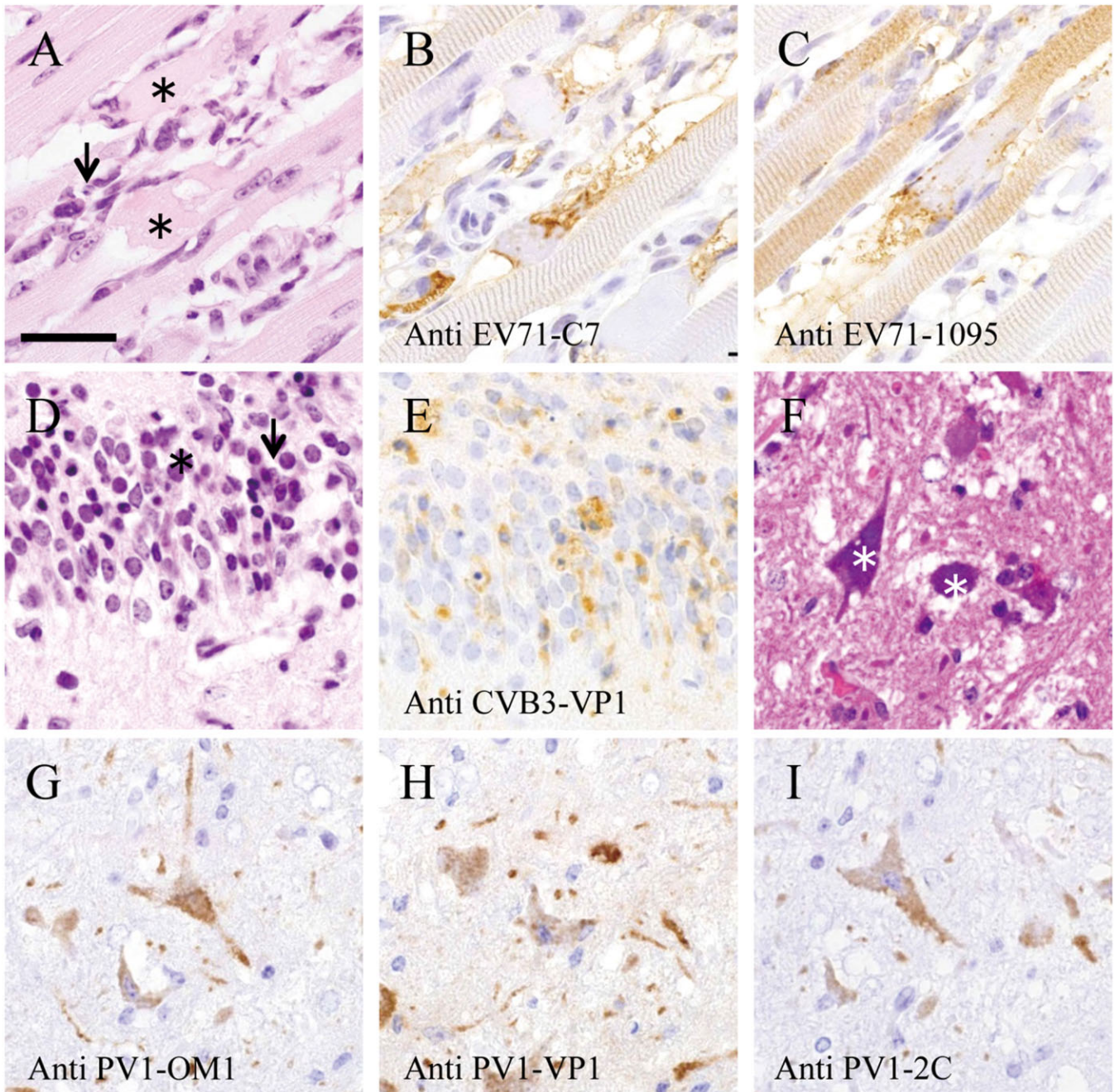


Fig. 3 Representative example of histopathological findings and immunohistochemical (IHC) analysis to detect enterovirus (EV) antigens in mice after experimental inoculation with EV. Three days after intracerebral inoculation with enterovirus A71 (EV71, SK-EV006/Malaysia 97), neonatal mice showed limb paralysis due to skeletal muscle degeneration (asterisk) with some inflammatory infiltration (arrow) (A). Anti-EV71-C7 (B) and anti-EV71-1095 (C) antibodies detected virus antigens in degenerative cells. Coxsackievirus B3 (CVB3, Nancy)-infected neonatal mice showed spastic paralysis and were moribund at Day 2 post-injection. Degenerated neurons (asterisk) and a very small inflammatory infiltrate (arrow) were seen in the hippocampus (D). The anti-CVB3-VP1 antibody detected virus antigens in cells in the lesion (E). Mouse virulent poliovirus type 2 (Lansing strain) caused flaccid paralysis in ICR mice at 2 days after intraspinal inoculation. Degenerated motor neurons (asterisk) are seen in the anterior horn of the lumbar spinal cord (F). The anti-PV1-OM1 (G), anti-PV1-VP1 (H) and anti-PV1-2C antibodies detected virus antigens in the cells (I). A, D, F, HE staining. B, C, E, G-I, IHC. Magnification, $\times 100$. Scale bar = 20 μm .

Table 3 Detection of viral antigens in neonatal ddY mice after intracerebral inoculation with enteroviruses

Inoculum			Sacrificed day for this study	Total number of mice	CNS		Skeletal muscle		Mucosal epithelium
Species	Serotypes	Virus titer (CCID ₅₀)/mouse			Brain	Spinal cord	Feet	Heart	
EV-A	CVA3	10 ^{6.9}	3 dpi	4	0†	0	4	2	0
	CVA4	10 ^{4.0}	2 dpi	4	0	0	4	1	0
	CVA6	10 ^{6.3}	2 dpi	4	0	0	4	2	2
	CVA10	10 ^{5.5}	3 dpi	4	0	0	4	0	4
	CVA16	10 ^{4.3}	6 dpi	4	0	3	4	0	0
	EV71	10 ^{5.8}	3 dpi	4	0	4	4	0	0
EV-B	CVA9	10 ^{5.5}	4 dpi	4	0	0	3	0	1
	CVB1	10 ^{7.5}	2 dpi	3	3	2	1	0	0
	CVB2	10 ^{5.2}	3 dpi	4	4	1	0	0	0
	CVB3	10 ^{6.1}	2 dpi	4	4	2	2	0	0
	CVB4	10 ^{5.8}	5 dpi	3	3	3	1	0	3
	CVB5	10 ^{7.3}	7 dpi	4	4	0	0	0	0

†Number of virus antigen-positive animals; dpi, days post-injection.

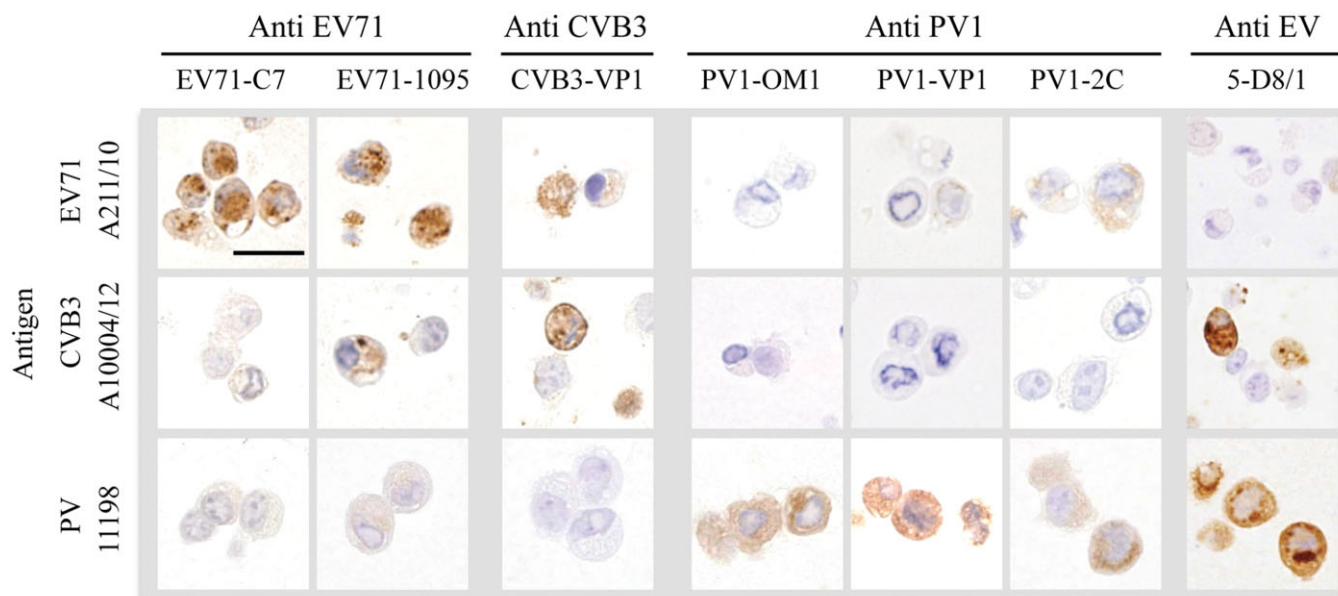


Fig. 4 Representative test panels of antibodies used for immunohistochemical (IHC) analysis of human enterovirus isolates. Rhabdomyosarcoma (RD) cells were inoculated with human isolates of enterovirus A71 (EV71, A211/10), coxsackievirus B3 (CVB3, A10004/12), or poliovirus (PV2, 11198) and then harvested on Day 1 post-inoculation. Formalin-fixed paraffin-embedded tissues were used for IHC. Magnification, $\times 100$. Scale bar = 20 μm

PJ161, PJ154, 11196 and 11198 and the Sabin1 strain showed differing degrees of homology with the PV1 Mahoney strain: PJ161, 10 sites of amino acid differences; PJ154, 11 sites; 11196, 70 sites; 11198, 72 sites; and Sabin1, seven sites).

DISCUSSION

The anti-EV71-C7, anti-EV71-1095 and anti-PV1-OM1 antibodies that we generated in a previous study were raised against denatured virus particles, and all recognized major virus-derived proteins.³⁴ Proteins can be made more

immunogenic by heating or by exposure to SDS.⁴⁴ EV harbors several group-common linear epitopes within its structural and non-structural proteins. Enzyme-linked immunosorbent assays using sera from EV-infected patients revealed that the most reactive peptides were derived from conserved regions at the amino-terminus of VP1.⁵⁰ VP1 mediates viral attachment to target cells and bears several neutralizing epitopes; however, it also shares epitopes with other EV. The anti-EV71-1095 antibody appears to recognize more linear epitopes on the VP1 antigen than the anti-EV71-C7 antibody, resulting in greater cross-reactivity with other EV (although the back-

Table 4 Detection of viral antigens from human isolates in cell blocks by immunohistochemistry using a panel of in-house polyclonal antibodies

Antigens		Antibodies									
Species	Serotypes	Strains	Major symptom	Year	Anti-EV71		Anti-CVB3		Anti-PV1		Anti-EV 5-D8/1
					EV71-C7	EV71-1095	CVB3-VP1	PV1-OM1	PV1-VP1	PV1-2C	
EV-A	EV71	A211/10	Fever	2010	+	+	+	-	-	-	-
		75/Yamagata/Japan 97	HFMD	2003	+	+	+	-	-	-	w
		C7/Osaka/Japan 97	Encephalitis	1997	+	+	+	-	-	-	w
		1095/Japan 97	HFMD	1997	+	+	+	-	-	-	w
		BrCr-A/39C-1tr	Aseptic meningitis	1970	+	+	+	-	-	-	w
EV-B	CVB3	A10004/12	Fever	2012	-	-	+	-	-	-	-
		A201/11	Herpangina	2011	-	-	+	-	-	-	+
EV-C	PV	11196 (KTS09-02)	AFP	2005	-	-	-	+	+	+	+
		11198 (BOS07-02)	AFP	2005	-	-	-	+	+	+	+
		PJ161 (Luzon-01-1)	AFP	2001	-	-	-	+	+	+	+
		PJ154 (Mindanao-01-1)	AFP/aseptic meningitis	2001	-	-	-	+	+	+	+
		Sabin1	Oral poliovaccine	(NIBSC)	-	-	-	-	-	-	-

†Non-specific signals were detected; shaded columns were for homologous antibodies/antigens in the serotypes. AFP, acute flaccid paralysis; HFMD, hand-foot-and-mouth disease; W, detected signals were weak.

ground staining was also higher). In addition, when we examined the reactivity of the antibodies against denatured virus particles, we noted differences in reactivity depending on the technique (IFA, WB or IHC) used. We also examined anti-GST-CVB3-VP1 and anti-GST-PV1-VP1 fusion protein antibodies, which recognize the capsid protein of VP1. These antibodies showed similar reactivity depending on the assay: IFA, WB and IHC. We also raised a polyclonal PV1 2C antibody against PV 2C, which is highly conserved among EV. Genetic analysis of the 2C amino acid regions suggest that the protein has many functions, including that of a serine protease inhibitor that regulates PV 3C protease activity during viral replication.⁵¹ The aim of the present study was to develop an antibody that could detect all serotypes of EV. Indeed, the anti-PV1 2C antibody recognized *EV-A*, *-B* and *-C* in the WB experiments; however, it only recognized PV and *EV-A* in the IHC analyses. In addition, the antibody generated a high background signal when used to examine monkey and human tissues (data not shown).

The commercial monoclonal anti-EV antibody (clone 5-D8/1), which is broadly cross-reactive with EV, recognizes an EV group-specific epitope located on VP1; ²⁴however, Hansson *et al.* recently reported that clone 5-D8/1 also recognizes two human mitochondrial enzymes: ATP synthase subunit beta (ATP5B) and creatine kinase B-type (CKBB). Cross-reactivity with the mitochondrial enzymes was prevented by blocking specimens with a solution containing excess ATP5B and CKBB.⁵² Another commercial anti-EV monoclonal antibody is also available, although it cross-reacts with rhinovirus, coronavirus, herpes virus and reovirus.⁵³ Animal tissue samples obtained after experimental infection are more similar to human specimens than cultured cells and are thus useful for evaluating non-specific binding. Here, we used paraffin-embedded tissue samples from neonatal mice infected with prototype virus or EV isolates (this was not possible for Echo because the virus does not infect mice). A common problem associated with polyclonal antibodies for IHC in tissue samples (both human and animal) is the high level of background staining due to non-specific binding or specific cross-reactions.⁵⁴ To solve this, we used powdered-acetone tissue or cultured cell preparations to block non-specific binding; we also purified the IgG fraction in an attempt to reduce non-specific binding.⁵⁴ These treatments mostly worked well, although higher antibody concentrations were needed in the assays (data not shown). Treatment with acetone powder was successful in reducing the non-specific binding of the anti-EV71 and PV antibodies when examining specimens from animals with EV71 and PV encephalitis after experimental infection.^{34,35}

We also examined the cross-reactivity of the six polyclonal antibodies with other picornaviruses, including

HPeV and SAFV. It is important to distinguish EV infection from HPeV and SAFV infections because HPeV and SAFV have been isolated from infants with gastroenteritis or respiratory illness, and from some patients with acute flaccid paralysis, aseptic meningitis, encephalitis, myocarditis or neonatal sepsis-like syndrome.^{55–63} Neither HPeV nor SAFV cross-reacted with the six in-house polyclonal antibodies. The current results showed that none of the six anti-EV polyclonal antibodies are specific for any particular serotype or species; however, the antibodies did not cross-react with other human picornaviruses. In short, the in-house antibodies are specific for the genus EV.

Since the HFMD outbreak of 1997 in Malaysia, EV71 epidemics have occurred cyclically in Asian countries.^{64–68} EV71 infection may result in neurological complications, aseptic meningitis and fatal encephalitis. In addition, CVB and Echo infections have been reported by several countries undertaking surveillance of acute flaccid paralysis; the effective control of these viruses will be important in the post-polio eradication era.^{69–71} Some countries have developed new vaccines against EV71 and neonatal mice are a candidate animal model for evaluating vaccine efficacy.^{72–74} Neonatal mice have been used for both virus isolation and neutralization studies to identify different EV. Small murine models of picornavirus infection are both convenient and useful for immunological analyses; however, the sensitivity of rodents to picornavirus infection is different from that of humans. Thus, it is necessary to understand differences in sensitivity between human and rodent models when studying the pathogenesis and development of vaccines and anti-viral agents. For instance, the pathogenic mechanism in neonatal mice in this study seems to be different from that in human cases of EV71-mediated severe encephalomyelitis. In addition, in EV71-infected neonatal mice, viral antigens were present in both muscle and the CNS; however, there is no evidence for virus infection of muscle in human cases.^{23,33} Thus, we need to carefully evaluate the neonatal mouse and mouse-adapted EV71 models.⁷⁵ The six in-house polyclonal antibodies described herein are very useful for evaluating the pathological dissimilarities between enterovirus infections in humans and experimental animals. Thus, these data will contribute to future studies of picornavirus infection.

Here, we used IFA, WB and IHC analyses to show that the six in-house polyclonal antibodies recognized antigens derived from the corresponding immunizing viruses. Although the specificity of the anti-EV antibodies for EV was low and they were not able to distinguish between different EV serotypes, they were specific for the EV genus and did not recognize other genera such as HPeV and SAFV. In addition, it should be noted that the detection of viral antigens in tissue specimens is dependent upon the phase of the infection. Thus, clinicians, virologists and

pathologists must collaborate to ensure a definitive diagnosis of picornavirus infection. Also, when tissue samples are available, IHC and PCR and/or ISH should be performed. In conclusion, the present study established a panel of in-house polyclonal antibodies suitable for the pathological diagnosis of picornavirus infections, such as CVA, CVB, Echo and PV. These results will help facilitate future diagnosis and research into the pathogenesis of EV infections.

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