

A murine oral enterovirus 71 infection model with central nervous system involvement

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Enterovirus 71 (EV71) infection causes a myriad of diseases from mild hand-foot-and-mouth disease or herpangina to fatal meningoencephalitis complicated with neurogenic pulmonary oedema. Its pathogenesis, especially the CNS involvement, is not clearly understood. The aim of this study was to set up a mouse EV71 infection model with CNS involvement. EV71 virus was administered orally to neonatal mice. The EV71-infected mice manifested a skin rash at an early stage and hind limb paralysis or death at a later stage. Immunohistochemical staining and virus isolation demonstrated that EV71 replicated in the small intestine, induced viraemia and spread to various organs. Kinetic studies showed that EV71 antigen was first detected in the intestine at 6 h, in the thoracic spinal cord at 24 h, in the cervical spinal cord at 50 h and in the brain stem at 78 h post-infection. Leukocyte infiltration was evident in the spinal cord and brain stem. Furthermore, EV71 virus could be transmitted to littermates within the same cage.

INTRODUCTION

Enterovirus 71 (EV71) is a human enterovirus in the *Enterovirus* genus of the *Picornaviridae* family. It is a major cause of hand-foot-and-mouth disease (HFMD) in young children. Following its initial identification in the USA in 1969, outbreaks have been reported in Australia, southeast Asia and Europe (Lum *et al.*, 1998; Schmidt *et al.*, 1974; Shindarov *et al.*, 1979; Wang *et al.*, 1999). Taiwan experienced four major epidemics in 1998, 2000, 2001 and 2002. Infection with EV71 is usually self-limiting, but may progress to cause aseptic meningitis, brainstem encephalitis and acute flaccid paralysis that is indistinguishable from poliomyelitis. A remarkable feature of this disease is the high mortality associated with brainstem encephalitis (26%) (Wang *et al.*, 1999). Most of the fatal cases occur in children less than 3 years of age. They rapidly develop progressive sympathetic hyperactivity, pulmonary oedema and/or haemorrhage and cardiopulmonary collapse (Ho *et al.*, 1999; Huang *et al.*, 1999; Liu *et al.*, 2000; Wu *et al.*, 2002).

The pathogenesis of EV71 infection, especially the CNS involvement, is not clearly understood. Little is known of the factors contributing to the manifestation of CNS symptoms. We have reported that neonatal mice can be infected with EV71 experimentally. Intraperitoneal inoculation of 10^8 p.f.u. of EV71 induced death of 1-day-old ICR mice in an age- and dose-dependent manner (Yu *et al.*, 2000). Since intraperitoneal injection is not the natural route for enterovirus infection, the application of this experimental infection model is limited. The objective of this study was to establish a murine EV71 model in which

mice are orally infected and manifest symptoms that mimic the clinical situation in humans.

METHODS

Cell cultures and mice. Vero and SK-N-SH cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, while Caco-2 cells were maintained in DMEM containing 20% FBS. Breeder mice of the ICR stock were purchased from Charles River Japan, Inc. (Atsugi, Japan). They were maintained on standard laboratory chow and water *ad libitum* in the animal facility of the Medical College, National Cheng Kung University, Tainan, Taiwan. The animals were raised and cared for according to the guidelines set up by the National Science Council of the Republic of China.

Enterovirus 71. Local isolates of EV71 (4643, 6360, 6367, 6321, 5811 and 5101) were obtained from the Virological Center, National Cheng Kung University, Tainan, Taiwan. These EV71 isolates were derived from different sources: EV71 4643, 6360 and 6367 were from fatal cases with CNS involvement; 6321 was from a non-fatal case with CNS involvement; and 5811 and 5101 were from patients with HFMD. The mouse-adapted strain was obtained from brain tissues of mice injected with EV71 4643 strain by intraperitoneal administration. The virus was further propagated in the Vero cell line before use. In some experiments, the Caco-2 cell line-adapted EV71 virus was used. EV71 4643 was propagated in the Caco-2 cell line. Virus stocks were collected by freezing and thawing three times. The titre of each virus pool was determined by the standard method of plaque assay on Vero cells (see below) and found to be 1×10^7 p.f.u. ml⁻¹. The sequence of the EV71 strains Tainan/4643/98 has been deposited in the GenBank database under accession number AF304458. (Yang *et al.*, 2001). The sequence of the mouse-adapted 4643 isolate has 99% homology with that of the parental 4643.

Plaque assay. Vero cells (1×10^5 cells per well) were plated onto a 24-well plate, incubated overnight and infected with 200 μ l of serially diluted virus suspension. After adsorption for 2 h, the virus suspension was replaced with DMEM containing 2% FBS and 0.5% methylcellulose (Sigma). The medium was removed at 72 h post-infection and the cells were stained with crystal violet solution (1% crystal violet, 0.64% NaCl and 2% formalin) (Wen *et al.*, 2003).

Infection of EV71 in Caco-2 and SK-N-SH cells. Caco-2 and SK-N-SH cells were infected with EV71 4643 or mouse-adapted EV71 at an m.o.i. of 1 for 1 h. After washing with PBS, cell cultures were incubated at 37 °C and 5% CO₂. Virus titre was determined at various intervals using Vero cells as described above.

Infection of EV71 in intestinal organ cultures. The organ culture of the small intestine was set up following the procedure described by Baumler *et al.* (1996). Briefly, a section of jejunum (1 cm) was dissected from a 1-day-old ICR mouse and placed in PBS. One end of the intestine was secured using a 6/0 braided silk suture (Chia Ho Silk Sutures Co., Taipei, Taiwan), while the other end was secured immediately after filling the lumen with 10 μ l of EV71 (2×10^5 p.f.u.). The organ cultures were incubated in DMEM containing 10% FBS at 37 °C for 24 h.

EV71 infection in mice. For oral infection, groups of three to six 1-day-old pups were intragastrically inoculated with EV71 or mouse-adapted EV71 (1×10^6 or 1×10^7 p.f.u. per mouse) using a plastic feeding tube after fasting for 8 h. The mice were monitored daily for clinical signs. In some experiments, groups of 7-day-old mice were intragastrically inoculated with 2×10^6 p.f.u. of mouse-adapted EV71 per mouse. For virus isolation, various organs were weighed, homogenized and centrifuged. Clarified supernatants were inoculated into Vero cells after a 10-fold dilution. The titre was determined as described above and expressed as p.f.u. per 10 mg protein. For the determination of viraemia, 50 μ l of blood sample was collected from each mouse at various times post-infection. The blood samples were diluted fourfold before infection of Vero cells. The titre was determined and expressed as p.f.u. ml⁻¹ blood. The thresholds of detection for tissue and blood were 0 and 4 p.f.u., respectively.

Histological and immunohistochemical analysis. Various tissue samples, including the intestines, spinal cord, heart and limb muscle, were removed from infected mice, embedded in OCT compound (Miles Inc.) and immediately frozen in liquid nitrogen. Four μ m cryosections prepared with a Leica CM 1800 were placed on

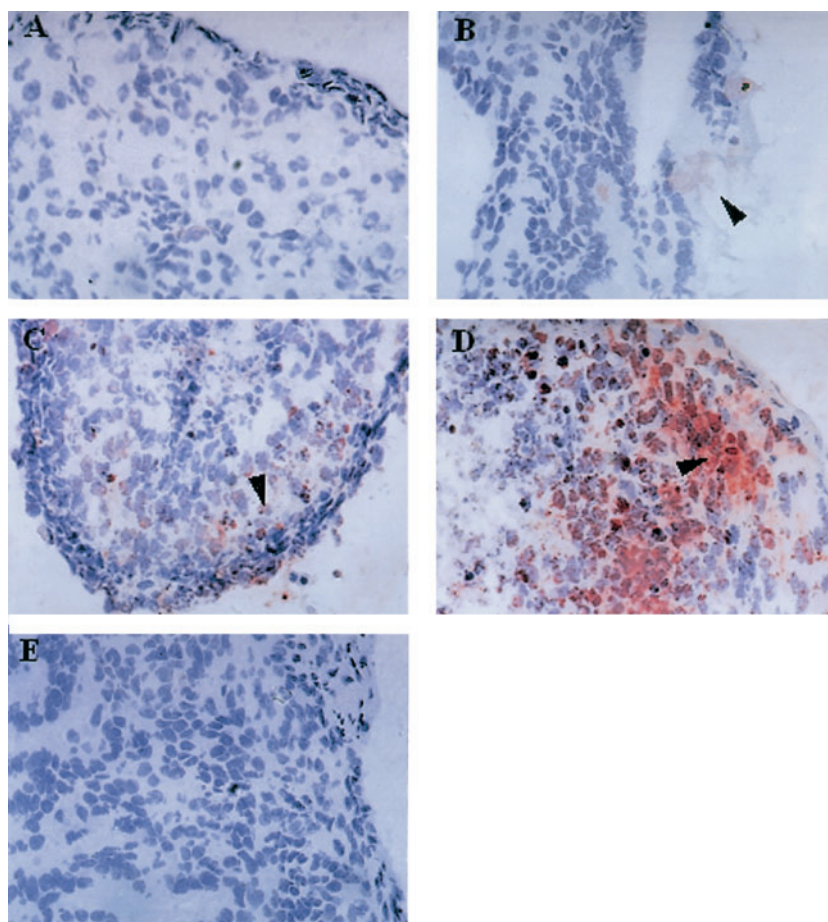


Fig. 1. Detection of EV71 infection in neonatal murine small intestine *ex vivo*. Intestine organ cultures (1 cm in length) were set up from 1-day-old ICR mice and incubated with medium alone (A), 2×10^5 p.f.u. of 4643 (B), 2×10^5 p.f.u. of Caco-2-adapted 4643 (C), or 2×10^5 p.f.u. of mouse-adapted 4643 (D) for 24 h. Cryosections were then prepared and stained with anti-EV71 VP1 antibody. The arrowhead indicates a positive staining. The antibody IgG1 isotype control for mouse-adapted 4643 infection is shown in (E).

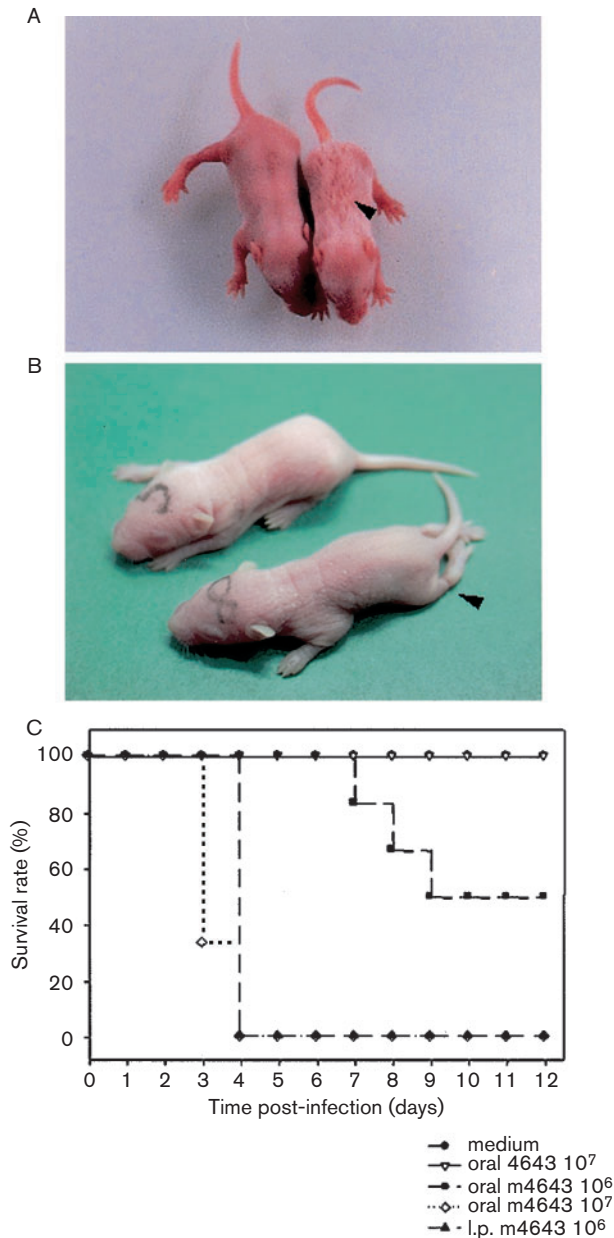


Fig. 2. Infection of mice with EV71 by the oral route. Groups of six 1-day-old ICR mice were orally inoculated with 1×10^6 p.f.u. EV71 per mouse. (A) A representative picture of skin lesions caused by clinical isolate 4643 at day 6 post-infection is shown. (B) A representative picture of hind limb paralysis caused by mouse-adapted 4643 at day 4 post-infection is shown. The mouse on the left-hand side of each figure is a naive age-matched control. (C) EV71-induced mortality. Groups of six 1-day-old ICR mice were inoculated with 10^6 or 10^7 p.f.u. EV71 (mouse-adapted 4643/m4643 or parental 4643) per mouse orally or intraperitoneally. Mortality was recorded every day. The percentage of survival is presented. One representation of three independent experiments is shown.

poly-L-lysine-coated glass slides and fixed with 3.7% paraformaldehyde in PBS. Endogenous peroxidase was inhibited by 3% H_2O_2 in PBS. EV71 was detected using mouse monoclonal anti-EV71 VP1 antibody (Chemicon International, Inc.). Vector M.O.M. agent was used in all incubation and washing steps to eliminate the background of mouse antibody on mouse tissues. The slides were then incubated with biotinylated anti-mouse antibody followed by Vectastain ABC-peroxidase complex (Vector Laboratories). A red-coloured peroxidase stain was developed using aminoethyl carbazole (Zymed Laboratories) substrate and counterstained with Mayer's haematoxylin (Merck). Mouse organs were fixed with 3.7% formaldehyde for 48 h and embedded in paraffin. Four μ m sections were made and stained with haematoxylin and eosin Y for morphological examination.

RESULTS

Replication of EV71 in Vero, Coca-2 and SK-N-SH cells

To determine the infectivity and tropism of EV71 in cell lines of different origins, polarized epithelial cell line Caco-2 and neuroblastoma cell line SK-N-SH, as well as the common kidney epithelial Vero cell line, were infected with each of several EV71 clinical isolates (4643, 6360, 6367, 6321, 5811 and 5101) at an m.o.i. of 1. Although there were large variations among different isolates in the three cell lines, in general, all the EV71 strains tested could replicate in Caco-2, SK-N-SH, or Vero cell lines (data not shown). No difference was found on the growth curve among the isolates from fatal cases with CNS involvement and those from patients with mild HFMD. Both 4643 and 6367 strains were derived from fatal cases, but the virion production of 4643 was higher than that of 6367 in the SK-N-SH cell line. Strain 5101 was isolated from HFMD, but its virion production was similar to that of 4643 in the SK-N-SH cell line. Thus, the virus growth curves in the various cell lines did not appear to illustrate any difference in virulence among these EV71 isolates. No significant correlation was found between tropism and susceptibility of cell lines to EV71 isolates from different categories of EV71-infected patients. However, 4643 grew better than the other isolates and so was chosen for use in the following experiments.

Replication of EV71 in intestinal organ cultures

As we aimed to develop a mouse model of EV71 infection via the oral route, we first tested whether EV71 could infect murine small intestine. Intestinal organ cultures (1 cm in length) were prepared from 1-day-old pups and infected with 2×10^5 p.f.u. EV71 in the lumens for 24 h. To ensure that infection had occurred, Caco-2-adapted 4643 and mouse-adapted 4643 were used in comparison with the clinical isolate of 4643. Immunohistochemical staining using anti-VP1 antibody showed that EV71 was present in the enterocytes of the organ cultures (Fig. 1). Under the same staining conditions, the most intense VP1 stain was noted in cultures infected with mouse-adapted 4643 rather than in those infected with Caco-2-adapted or the

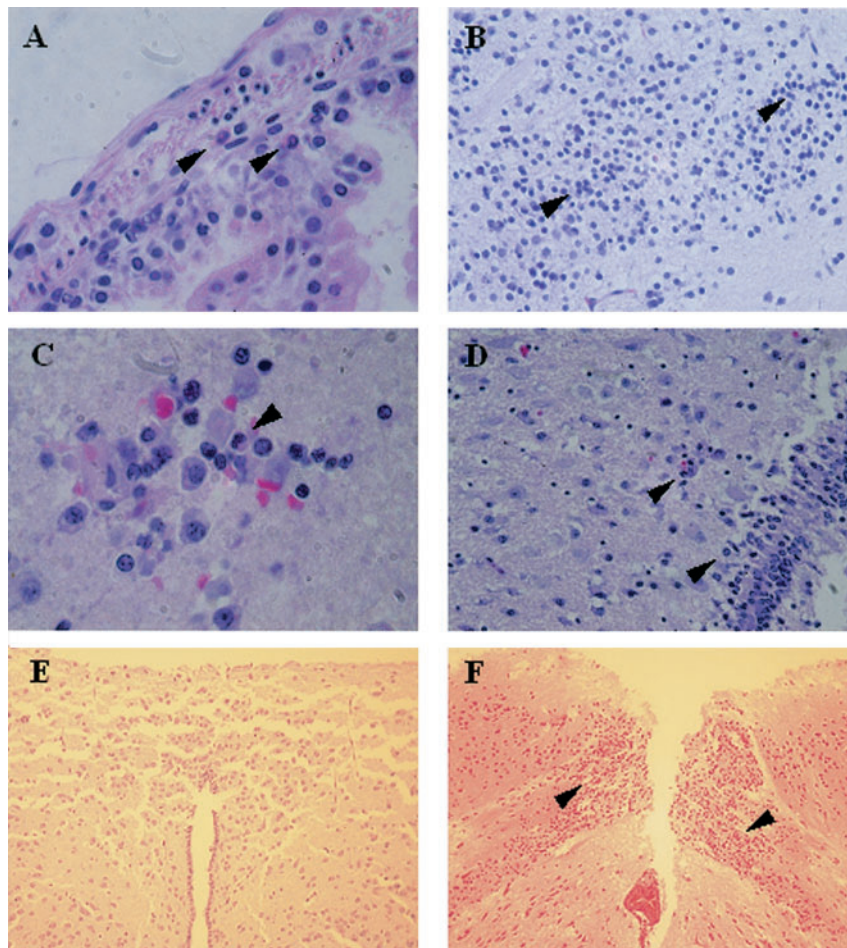


Fig. 3. Histological changes in EV71-infected mice. Groups of three 1-day-old ICR mice were orally inoculated with 1×10^7 p.f.u. of mouse-adapted EV71 per mouse. Tissue sections prepared at 3 days post-infection were stained with haematoxylin and eosin. Polymorphonuclear cell infiltration (arrowheads) was observed in muscular layers of the intestine (A). Mononuclear cell infiltration (arrowheads) was observed in the brain (B), spinal cord (C) and brain stem (D). Brain tissue of mock-infected (E) and EV71-infected (F) mice is shown at lower magnification ($\times 100$).

original 4643. This indicated that EV71 can infect the epithelial cells of the small intestine.

EV71 infection in mice

Since EV71 can infect intestinal epithelial cells *ex vivo*, we further tested its *in vivo* infectivity by the oral route. One-day-old ICR mice were orally inoculated with 1×10^6 p.f.u. 4643 or mouse-adapted 4643 after fasting for 8 h. In one particular experiment, at day 2 post-infection, 10/11 EV71 4643-infected mice (90 %) developed skin lesions, which were characterized as desquamation followed by cicatrization. The hairless lesions persisted throughout the observation period until day 10, while the mice otherwise remained healthy and grew normally (Fig. 2A). Similar results were observed in three other experiments, in which 15/17 mice manifested skin lesions. On the other hand, mouse-adapted-4643-infected mice developed more severe symptoms. Skin lesions occurred

at day 2, followed by movement disorientation at day 3–6 post-infection. In general, mice developed hind limb paralysis at day 4–7 and then died at day 7–9 post-infection (Fig. 2B). In a total of six experiments, 22/37 orally infected mice developed paralysis and approximately 50 % of the paralysed mice died. The clinical manifestation induced by oral and intraperitoneal inoculation was slightly different. Mice infected by intraperitoneal injection did not manifest skin lesions, although they developed paralysis and died with the same challenge dose. The orally infected mice had a longer survival time than those with intraperitoneal injection. The LD₅₀ of different routes was also different. For mouse-adapted 4643, the LD₅₀ for the oral route was 1×10^6 p.f.u., while a dose of 1×10^7 p.f.u. resulted in a 100 % death rate. The LD₅₀ for the intraperitoneal route was 1×10^5 p.f.u. Mouse-adapted 4643 was thus more virulent than parental 4643 in this murine oral infection model (Fig. 2C).

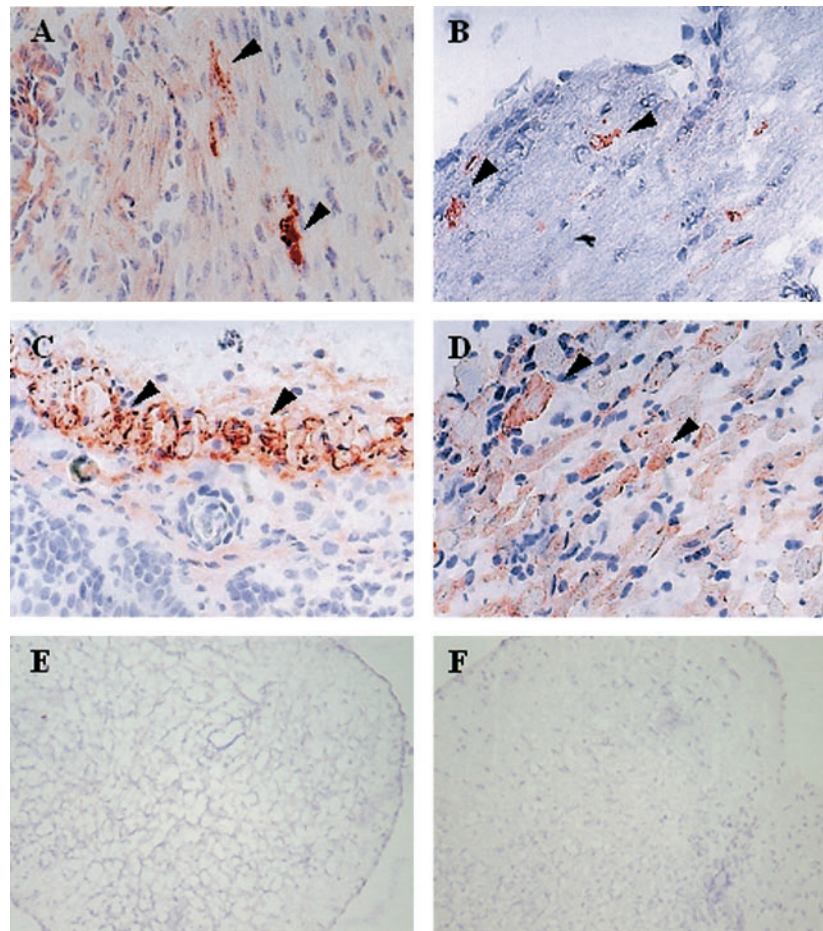


Fig. 4. Organ distribution of VP1 antigen in EV71-infected mice. Groups of three 1-day-old ICR mice were orally inoculated with 1×10^7 p.f.u. of mouse-adapted EV71 per mouse. Cryosections of the heart (A), spinal cord (B), skin (C) and limb muscle (D) were stained with anti-EV71 VP1 antibody at day 3 post-infection. The arrowheads indicate positive staining. The spinal cord of naïve mouse was used as a negative control (E). The antibody isotype control for spinal cord is shown in (F).

Histological observations in EV71-infected mice

Histological examinations revealed leukocyte infiltration in various tissues after EV71 oral infection. Neutrophil infiltration was found in the muscular layers of the small intestines while mononuclear cell infiltrations were evident in the brain, spinal cord and brain stem (Fig. 3A–D). The lymphocyte infiltration was more extensive in the brains of infected mice compared with the mock-infected control (Fig. 3F vs E). The VP1 protein of EV71 could be detected in these areas with anti-VP1 antibody. Heart, spinal cord, skin and limb muscle exhibited the strongest staining, indicating vigorous virus replication (Fig. 4). To explore the route of virus spread *in vivo*, particularly in the CNS, organs including small intestine, skin, brain stem and spinal cord were collected at different time intervals post-infection and stained with anti-VP1 antibody. Kinetic analysis showed that the VP1 antigen first occurred in the intestine at 6 h post-infection, gradually decreasing thereafter. The VP1 antigen was not found in the skin until 12 h post-infection and persisted thereafter. Interestingly, the VP1 antigen was

first detected in the thoracic spinal cord at 24 h post-infection, then on the cervical or lumbar spinal cord at 50 h post-infection. EV71 antigen was not detected in the brain stem until 78 h post-infection. Fig. 5 shows the earliest time point that VP1 was detected in tissue of intestine, skin, spinal cord and brain stem, and the results are summarized in Table 1. To demonstrate further how EV71 can replicate *in vivo*, blood and various organs were cultured for the presence of EV71. As shown in Fig. 6(A), there were two phases of viraemia, first at 6 h post-infection, then at 24 h and onward. At day 3 post-infection, EV71 could also be cultured from various tissues, of which skin and muscle contained the highest viral load (Fig. 6B).

EV71 transmission between littermates

In our experimental set-up, EV71-infected pups were reared in the same cage as the non-treated littermate controls. During the 21 day observation period, the orally infected pups showed paralysis at days 4–7 post-infection.

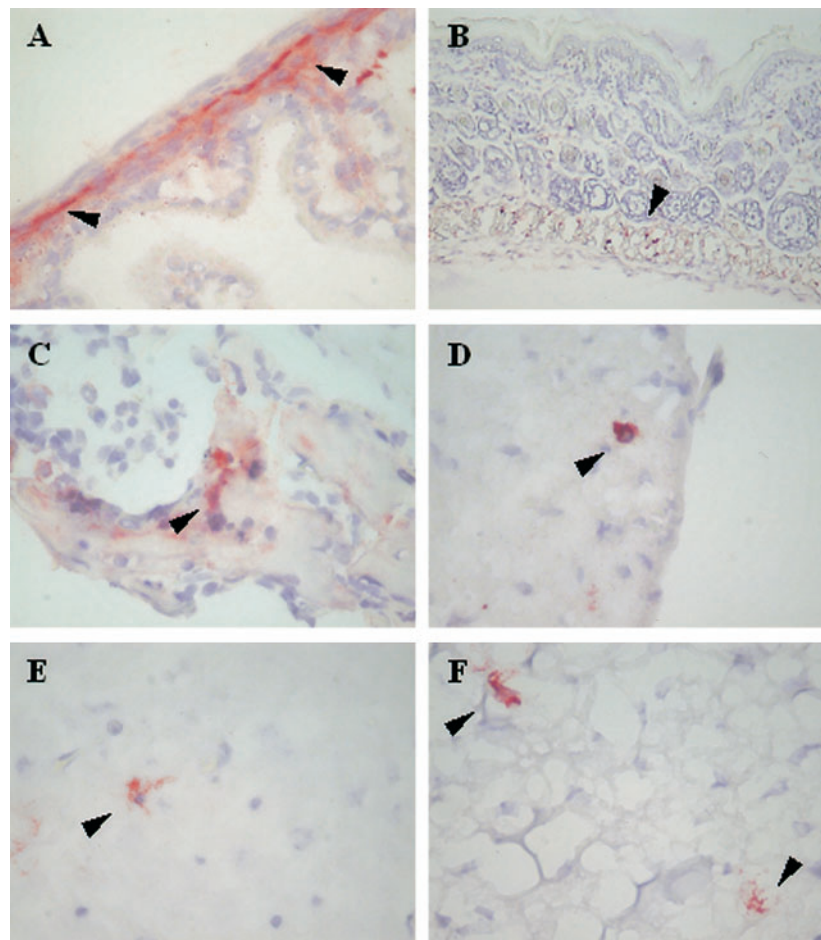


Fig. 5. Kinetic expression of EV71 in spinal cord after oral infection. Groups of three 1-day-old ICR mice were orally inoculated with 1×10^7 p.f.u. of mouse-adapted EV71 per mouse. Cryosections of various organs were prepared and stained with anti-EV71 VP1 antibody at various times post-infection. Positive stains of the first time point at which VP1 antigen can be detected are shown: (A) intestine, 6 h; (B) skin, 12 h; (C) thoracic spinal cord, 24 h; (D) cervical spinal cord, 50 h; (E) lumbar spinal cord, 50 h; (F) brain stem, 78 h. The arrowheads indicate positive staining. The results are summarized in Table 1.

Table 1. Summary of EV71 VP1-positive staining in different tissues of EV71-infected mice.

Tissue	Naïve	Time (h) post-infection with EV71				
		6	12	24	50	78
Intestine	—	+	+	+	—	—
Colon	—	—	—	—	—	—
Brain stem	—	—	—	—	—	+
Spinal cord						
Cervical	—	—	—	—	+	+
Thoracic	—	—	—	+	+	+
Lumbar	—	—	—	—	+	—
Skin	—	—	+	+	+	+

Surprisingly, approximately 80% ($n=11$) of the non-treated littermate controls also showed a mild skin rash at days 4–6 post-experiment, even though the numbers showing desquamation and cicatrization were less than those of the EV71-infection experiment. The VP1 antigen was stained and found on the small intestine of the non-treated controls at 12 h, but not at 6 h post-experiment (Fig. 7). This demonstrated that EV71 was present in the non-treated controls and that, while there was a lag of EV71 transmission from orally infected pups to the non-treated littermate control, nevertheless EV71 could be transmitted between littermates, probably either through the faecal–oral route or by close contact.

DISCUSSION

EV71 infection has become a severe health problem in Taiwan. More than 129 000 children were infected during

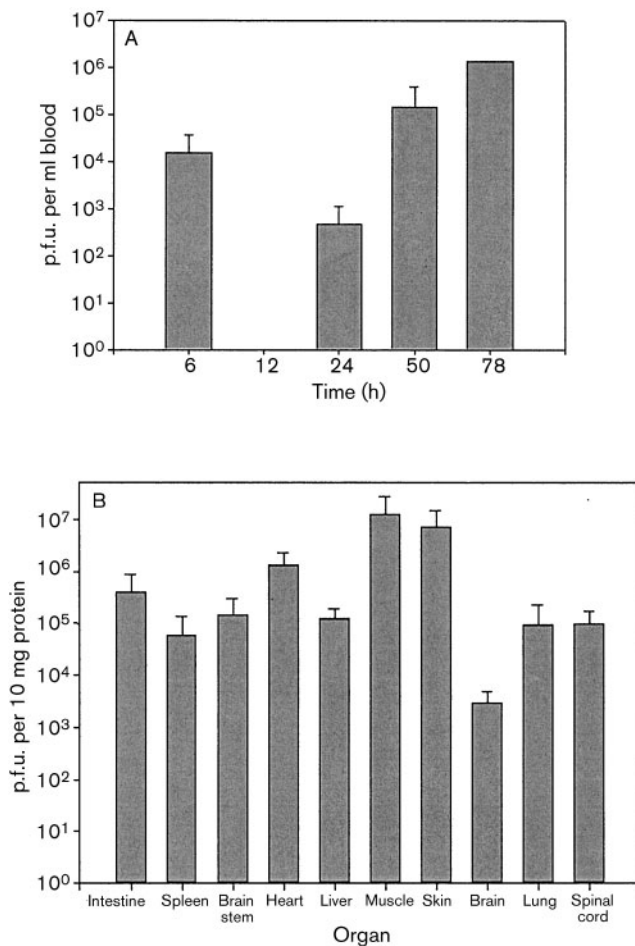


Fig. 6. Culture of EV71 from various organs of EV71-infected mice. Groups of four 1-day-old ICR mice were orally inoculated with 10^7 p.f.u. of mouse-adapted EV71 per mouse. Blood samples were collected at various times post-infection (A) and from various organs at day 3 post-infection (B). Virus titre was determined and expressed as p.f.u. per ml blood or p.f.u. per 10 mg protein. One representation of three independent experiments is shown.

the 1998 outbreak and 405 children were hospitalized due to HFDM-associated meningitis, encephalitis, or acute flaccid paralysis. Of these, 78 died (Ministry of Health, 1998). Brain stem encephalitis was the cardinal feature of these EV71 outbreaks, which progressed abruptly to neurogenic shock and neurogenic pulmonary oedema that were indicative of poor prognosis. The pathogenesis of the CNS involvement in EV71 infection is not known. We report here that EV71 can infect mice experimentally via oral inoculation. After oral inoculation, the virus initially replicates in epithelial cells of the alimentary tract and subsequently spreads to the circulation and various organs including heart, muscle, skin, liver and lung. Infected mice develop neurological symptoms and EV71 can be isolated from the brain and spinal cord. This model mimics the

clinical EV71 infection with CNS involvement and should shed light on the study of EV71 pathogenesis in the CNS.

We, along with other investigators, have reported the experimental infection of EV71 in mice (Wu *et al.*, 2001; Yu *et al.*, 2000). Intraperitoneal or intracerebral administration of EV71 in suckling mice resulted in death. When the virus was administered intraperitoneally in the neonatal mice, the mice died in an age- and dose-dependent manner at day 7–10 post-injection. In an attempt to develop an EV71 infection model to mimic the clinical infection via the oral route, we first demonstrated that EV71 could infect epithelial cells in either the Caco-2 epithelial cell line or in small intestine organ cultures. Although various EV71 clinical isolates could replicate in cell lines such as Caco-2 or SK-N-SH, the replicative activity of the mouse-adapted EV71 was higher than those of the original isolate or the Caco-2-adapted strain. In suckling mice, oral administration of clinical isolate 4643 only induced mild skin lesions, whereas mouse-adapted 4643 caused hind limb paralysis. The mouse adaptation of EV71 increases its virulence in mice with a higher expression of VP1 in the small intestine and a higher mortality. The mouse adaptation of EV71 seems to increase virus replication and enhance cell damage. The adaptation procedure was repeated four times and it was found that more adaptation caused more virulence in mice (Fig. 4, and unpublished observations).

In poliovirus infection, the virus first infects the gut, replicates in the epithelial cells lining the gut and is then released in the blood. Disseminated virus then replicates in skeletal muscle cells, finally reaching the peripheral nerves and then spreading to the CNS. Virus replication in skeletal muscle maintains a persisting viraemia. Poliovirus enters the CNS, probably through either penetration of the blood–brain barrier or transmission via peripheral nerves using active retrograde axonal transport (Crotty *et al.*, 2002; Ohka & Nomoto, 2001; Ren & Racaniello, 1992; Yang *et al.*, 1997). Paralytic poliomyelitis occurs as a result of neuronal destruction. In the reovirus serotype 3 infection in the newborn mouse model, reovirus first replicates in the lymphoid tissues of the gastrointestinal tract, then rapidly spreads to the mesenteric lymph nodes and produces viraemia (Mann *et al.*, 2002; Morrison *et al.*, 1991). Within 2 days of inoculation, viral titre increases in skeletal muscle and other extraneural sites and shortly thereafter is detected in the CNS. Viral antigen is detected by staining on the neurons of the dorsal motor nucleus of the vagus nerve in the brain stem. Reovirus enters a host via the lymphoid tissue of the alimentary tract, directly infecting nerves present in the muscle wall of the tract, and is then transported by them to the CNS. Thus, it spreads from the intestine to the CNS by vagus nerve fibres. In EV71 infection, our study has shown that EV71 initially replicates in the small intestine, followed by viraemia. The EV71 VP1 antigen was first detected in the thoracic segment of the spinal cord, later in the cervical segment of the spinal cord and then in the brain stem. EV71 might use the active

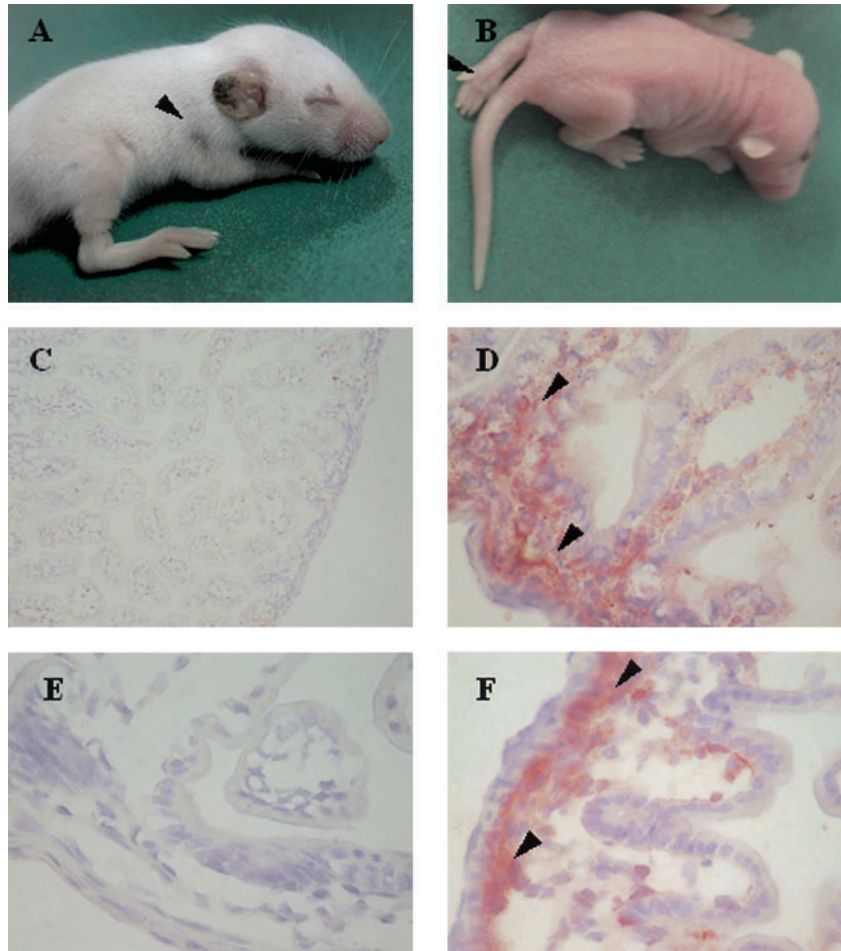


Fig. 7. Transmission of EV71 between littermates. Half of a group of 1-day-old ICR mice ($n=6$) were orally inoculated with mouse-adapted EV71 (10^6 p.f.u. per mouse) and reared in the same cages by the same mother with the other half of non-treated cagemates. Skin lesions developed on the non-treated mice: (A) shows the picture of one representative mouse at day 9 post-treatment. In contrast, EV71-treated mice exhibited paralysis: (B) shows the paralysis of one representative mouse at day 5 post-inoculation. Immunohistochemical staining of VP1 was not observed at 6 h (C), but was evident at 12 h (D) in the intestine of the non-treated littermate control. Anti-VP1 staining was detected at 6 h on the intestine of the EV71-inoculated mice (F). The antibody isotype control for the intestine of EV71-inoculated mice is shown in (E). The arrowheads indicate positive staining.

retrograde axonal transport system to enter the CNS, similarly to poliovirus. Of course, trafficking across the blood–brain barrier or by the peripheral vagus nerve is not excluded. The route by which EV71 spreads into the CNS is the subject of ongoing investigations. This example of CNS infection via the oral route provides a model to study the clinical manifestation of brain stem encephalitis, autonomous nerve system dysregulation and neurogenic pulmonary oedema caused by EV71 in humans (Huang *et al.*, 1999; Liu *et al.*, 2000; Wang *et al.*, 1999).

Furthermore, we found that EV71 can be transmitted between treated and non-treated littermates reared in the same cage. One-day old mice developed paralysis after oral inoculation of EV71. Surprisingly, mock-control mice

developed mild skin lesions, indicating that EV71 might be shedding from the gut of treated mice and then being transmitted to their cagemates through contact with infected faeces. Anti-EV71 antibodies were present in lactating dams after nursing a litter of infected pups. The pups of the next litter became resistant to EV71 challenge (unpublished observations). This suggests that infective EV71 was released from the experimentally infected mice and transmitted to the dams and their siblings and that the anti-EV71 antibodies were passively transferred from the dams to protect the next generation of pups. This oral-to-faecal transmission mimics clinical EV71 infection. Moreover, the questions of whether respiratory droplets can transmit EV71 and by what molecular mechanism EV71 virus is spread to the CNS can be investigated by this

model (Ho, 2000; McMinn, 2002). Intravenous immunoglobulin has been used clinically to treat severe cases of EV71 infection with variable results – the earlier the administration, the better the protection. This infectious murine model can be used for evaluation of the anti-EV71 antibody therapy. Moreover, it can also be used as an efficacy test for vaccine and anti-EV71 drug development.

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