The Distribution of Inflammation and Virus in Human Enterovirus 71 Encephalomyelitis Suggests Possible Viral Spread by Neural Pathways

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Abstract
Previous neuropathologic studies of Enterovirus 71 encephalomyelitis have not investigated the anatomic distribution of inflammation and viral localization in the central nervous system (CNS) in detail. We analyzed CNS and non-CNS tissues from 7 autopsy cases from Malaysia and found CNS inflammation patterns to be distinct and stereotyped. Inflammation was most marked in spinal cord gray matter, brainstem, hypothalamus, and subthalamic and dentate nuclei; it was focal in the cerebrum, mainly in the motor cortex, and was rare in dorsal root ganglia. Inflammation was absent in the cerebellar cortex, thalamus, basal ganglia, peripheral nerves, and autonomic ganglia. The parenchymal inflammatory response consisted of perivascular cuffs, variable edema, neuronophagia, and microglial nodules. Inflammatory cells were predominantly CD68-positive macrophage/microglia, but there were a few CD8-positive lymphocytes. There were no viral inclusions; viral antigens and RNA were localized only in the somata and processes of small numbers of neurons and in phagocytic cells. There was no evidence of virus in other CNS cells, peripheral nerves, dorsal root autonomic ganglia, or non-CNS organs. The results indicate that Enterovirus 71 is neuronotropic, and that, although hematogenous spread cannot be excluded, viral spread into the CNS could be via neural pathways, likely the motor but not peripheral sensory or autonomic pathways. Viral spread within the CNS seems to involve motor and possibly other pathways.

Key Words: CNS infection, Enterovirus 71, Human encephalomyelitis, Immunohistochemistry, In situ hybridization.

INTRODUCTION
Enterovirus 71 (EV71) is a picornavirus belonging to the same group of human enteroviruses that includes poliovirus and coxsackievirus (1). Many epidemics of EV71-associated hand, foot, and mouth disease, complicated or uncomplicated by neurologic disease, have been documented worldwide (2–6). The neurologic complications are varied and may manifest as aseptic meningitis, acute flaccid paralysis, and, more seriously, as a fatal encephalomyelitis or rhombencephalitis (3, 5, 7–9). Nonetheless, neurologic disease is relatively rare, and the mechanisms for tissue damage and the mode of viral spread into and within the central nervous system (CNS) are still not fully understood.

Magnetic resonance imaging (MRI) of the CNS in patients with encephalomyelitis has demonstrated that the distribution of lesions in EV71 encephalomyelitis seemed to be distinct and rather stereotyped (3, 10, 11). Lesions were found mainly in the medulla, pontine tegmentum, midbrain, and dentate nucleus of the cerebellum. Anecdotal reports from a few autopsies seem to support this and indicate, in addition, that other parts of the brain (e.g., hypothalamus and cerebral cortex) may also be inflamed (12–14). Interestingly, in some patients with severe neurologic disease, acute lesions revealed on brain MRI have been reported to disappear completely on recovery (11).

Detailed neuropathologic analysis of the features and distribution of inflammation have not been published and may offer insight into the pathogenesis of the neurologic disease. There is some evidence that neurologic disease has resulted from encephalitis, including immunohistochemical and ultrastructural evidence for direct neuronal infection as a cause of tissue damage (6, 12, 13, 15–18). It is possible that other CNS cell types can be targets for infection, but to date, there is no published information on this question. Little is also known about viral presence in non-CNS lymphoid and nonlymphoid organs.

The mechanism for viral spread into the CNS after the presumed viremic phase after fecal-oral transmission is not fully understood. Possible retrograde spread of the virus through
peripheral nerves has been proposed (3, 13, 14). In recent years, mouse models for poliovirus and EV71 have suggested that for both enteroviruses, neural pathways as a means for viral spread into the CNS may have a larger role than previously thought (19, 20). Moreover, the possible involvement of the autonomic and sensory nervous system in neural transmission in human infection has not been investigated.

The purpose of this study is to investigate the CNS cellular targets of EV71 infection and the nature and distribution of the CNS inflammatory response in a series of autopsy cases using light microscopy on small and large brain sections, immunohistochemistry (IHC), and in situ hybridization (ISH). On the basis of these findings, we propose that in human EV71 encephalomyelitis, viral spread into and within the CNS can be by neural pathways.

MATERIALS AND METHODS

Preparation of Cell Culture and Mouse Tissue Controls

Enterovirus 71 was inoculated into Vero cells at a multiplicity of infection of approximately 1 and fixed with 10% buffered formalin after 48 hours. The virus was previously isolated from a fatal human case of EV71 encephalomyelitis and obtained from the Department of Medical Microbiology, University of Malaya. Harvested cells were routinely processed and embedded in paraffin as a positive control in IHC and ISH to detect EV71 antigens and RNA, respectively. Mock-infected cells (negative controls) were also prepared in the same manner as for infected cells. One-day-old ICR suckling mice (n = 7) were inoculated intracerebrally with the same stock virus contained in 0.01 mL of EV71 Vero cell culture lysate. After 5 days, the mice were killed, and brains and hindlimb muscles were dissected out, fixed in 10% buffered formalin, and routinely processed and embedded in paraffin blocks to serve as a second positive control. Tissues from 2 uninfected mice were prepared in the same way as negative controls. Ethical clearance was obtained from the Animal Care and Use Committee, Faculty of Medicine, University of Malaya.

Preparation of Human Tissues

A total of 7 cases of fatal EV71 encephalomyelitis were studied (Table). Brain, spinal cord, and other major non-CNS tissues and autonomic and sensory nervous system in neural transmission thought (19, 20). Moreover, the possible involvement of the spread into the CNS may have a larger role than previously for both enteroviruses, neural pathways as a means for viral

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age/Sex</th>
<th>Clinical Presentation and Course</th>
<th>Autopsy Tissues Available for Study</th>
<th>Positive Viral Culture</th>
<th>IHC and ISH results in CNS Tissues*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36 mo/male</td>
<td>Lethargy, cough, and sore throat for 3 d before admission with tachypnea and cardiac arrest; died 6 h later. Chest x-ray showed diffuse pulmonary edema</td>
<td>CNS tissues* and non-CNS tissues†</td>
<td>Yes</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>36 mo/male</td>
<td>Lethargy, shortness of breath, fever, and cough for 3 d before admission. He was found to have cyanosis, tachypnea. Developed hypotension and bradycardia and died 2 h after admission. Chest x-ray showed evidence of pulmonary edema</td>
<td>CNS tissues and non-CNS tissues</td>
<td>Yes</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>15 mo/male</td>
<td>Cough, sore throat, tachypnea, sweating, cold extremities, and tachycardia before admission. Died 2½ h later. Had rashes on hands and feet. Chest x-ray showed diffuse pulmonary edema</td>
<td>CNS tissues and non-CNS tissues</td>
<td>Yes</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>48 mo/female</td>
<td>Fever and vomiting for 3 d associated with oral ulcers and lower-limb weakness. Just after admission, she became tachypnoeic then deteriorated rapidly to respiratory arrest. Died 2½ h later</td>
<td>CNS tissues and non-CNS tissues</td>
<td>Yes</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>13 mo/female</td>
<td>Fever, rash on arms and legs, lethargy, and poor feeding for 2 d. Admitted on the third day with cyanosis and tachypnea and died soon after from &quot;viral myocarditis.&quot; Maculopapular rash on sole of feet but no mouth ulcers</td>
<td>Brain, lung, and liver</td>
<td>NA</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>21 mo/male</td>
<td>Fever and vomiting for 2 d. Admitted to hospital on the third day with extreme lethargy, cold extremities, sweating, small ulcers on the hard palate, and lung crepitations. Died after 4 d of illness of &quot;viral myocarditis with cardiogenic shock.&quot;</td>
<td>CNS tissues and non-CNS tissues‡</td>
<td>NA</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>18 mo/male</td>
<td>Low-grade fever for 2 days and had 3 seizures. At the hospital emergency department, the patient had a cardiac arrest</td>
<td>CNS tissues and non-CNS tissues§</td>
<td>NA</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* Brain and spinal cord; spinal cord tissues included attached peripheral nerves.
† Included liver, lung, heart, spleen, thymus, pancreas, and kidney; in addition, intestine only available in Cases 1, 3, and 4; skeletal muscle only available in Cases 1 and 4; tonsil only available in Case 2; thyroid only available in Cases 1, 2, and 4.
‡ All non-CNS tissues available as stated above except thymus and pancreas.
§ All non-CNS tissues available as stated above except kidney.
† Neurohistochemistry; ISH, in situ hybridization; NA, not available.
large brain tissue sections were overlaid with 4-mm² square and examined by light microscopy. Blocks of test and control tissues and selected large blocks. Standard IHC protocol as described previously for EV71 was 10 minutes in sodium citrate buffer of pH 6.0. A modified series when positive results were obtained for IHC and ISH. These cases were included in this series when positive results were obtained for IHC and ISH.

All of the tissues were fixed in 10% buffered formalin for at least 2 weeks before trimming into small blocks, routine processing, and paraffin embedding. The Table summarizes the cases and the tissues examined. In addition, large brain sections of the cerebrum and cerebellum were done on the 5 cases autopsied in the University of Malaya. For this purpose, 1-cm coronal slices of cerebral hemispheres were cut after separation from the midbrain. The cerebellum with the brainstem attached was cut horizontally and perpendicularly to the long axis of the brainstem at 1-cm thickness at the midpons level. The large brain sections were processed manually during 2 to 3 weeks as for routine processing except that each change of the processing solution was spread out equally during this period.

Preparation of Probes for ISH

Digoxigenin (DIG)-labeled DNA probes were generated from a 500-bp nucleotide generated from a reverse transcriptase (RT)-polymerase chain reaction (PCR) procedure using the Access RT-PCR kit (Promega, Madison, WI) and published primers (22) to the 5-nontranslated region of EV71. The 1-step RT-PCR was performed in a DNA thermocycler (Perkin Elmer, Waltham, MA) under the following conditions: 1) 60 minutes at 37°C then 2 minutes at 94°C; 2) 1 minute at 94°C, 1 minute at 55°C, and 1 minute 72°C for 35 cycles; and 3) 5 minutes at 72°C. The approximately 500-bp PCR fragments were purified with a High Pure PCR purification kit (Boehringer Mannheim, Mannheim, Germany) and cloned into the pGEM-T easy vector (Promega) according to the manufacturer’s protocol. Plasmids containing the correct insert in either orientation were used as a template to produce DIG-labeled DNA probes by incorporating DIG-11-deoxyuridine triphosphate nucleotide (Boehringer Mannheim) in a separate PCR reaction (PCR condition was the same as described in the previous sentences except that the RT step was omitted). The DNA probes were purified with the High Pure PCR purification kit (Boehringer Mannheim).

Riboprobes were produced by linearizing the plasmids containing inserts in 2 orientations using the SpeI restriction enzyme (Promega) according to the manufacturer’s protocol. In this way, sense and antisense riboprobes were produced using a DIG RNA labeling kit (Roche, Mannheim, Germany) according to the manufacturer’s instruction. Synthesized riboprobes were purified as before.

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In Situ Hybridization

In situ hybridization was performed on all small tissue blocks as previously described, with minor modifications (23, 24). The tissue slides were deparaffinized, hydrated, and treated with 0.2 N HCl before digestion with 100 μg/mL proteinase K at 37°C for 20 minutes. Specimens were immersed in 0.2% (wt/vol) glycine in 0.15 mol/L phosphate-buffered saline (PBS) and washed with PBS. The sections were then prehybridized with 100 μL prehybridization mixture containing 6× saline sodium citrate, 45% formamide, 5× Dendhardt solution, and 100 μg/mL denatured salmon sperm DNA, and incubated for 30 minutes at 42°C for DNA probes (45°C for riboprobes). Next, the sections were hybridized with 50 μL of hybridization solution incorporating 10% dextran sulfate and 2 μg/mL of probe at 95°C for 10 minutes (this heating step was omitted for riboprobes) and then incubated overnight at 42°C for DNA probes (45°C for riboprobes).

After hybridization, slides were washed in 6× saline sodium citrate at 42°C, 2× saline sodium citrate, Tris-NaCl buffer for 5 minutes and blocked with 1% blocking solution (Boehringer Mannheim) for 30 minutes at room temperature. Hybridization was detected by anti-DIG Fab fragments linked to alkaline phosphatase (Boehringer Mannheim) and a color reaction obtained when nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim) substrate was applied. The reaction was stopped after 5 minutes by immersing in TRIS-EDTA buffer, and the slide was counterstained with Mayer hematoxylin. Duplicate ISH assays were also performed on all control tissues, as was used in the IHC. Additional negative controls used in the ISH assay included RNAase-treated, EV71-infected tissue sections and an assay that omitted the probe in the procedure.

RESULTS

Distribution and Histopathologic Features of Inflammation

Inflammation was observed in many parts of the CNS but always in a consistent, stereotyped distribution and intensity. More or less equally intensely inflamed areas

FIGURE 1. (A) Whole brain coronal sections of cerebral hemispheres with distribution of inflammation indicated by black dots (<half-field inflamed) and circles (<half-field inflamed), and viral antigens by stars. In the 3 separate areas where viral antigens were detected, only a total of approximately 5 neuronal bodies and/or their processes were positive. Inflammation was most intense around the hypothalamus area and least intense in the cerebral cortex, including the motor cortex (arrows). (B) Whole brain coronal sections of cerebral hemispheres (at the level of the splenium) with distribution of inflammation indicated by dots and circles. Immunohistochemistry for viral antigens was not performed in these sections. (C) Horizontal section of cerebellum and midpons showing the distribution of inflammation (dots and circles), but no viral antigens were detected. Inflammation was most intense in the tegmentum and dentate nucleus. The cerebellar cortex and anterior pontine nuclei areas were uninflamed.

FIGURE 2. Parenchymal inflammation (arrows) and perivascular cuffing in the inferior olivary nucleus area of the medulla (A). In more severely inflamed areas (B), there may be varying degrees of edema (*) and neuronophagia (C, arrows). More subtle inflammation in the motor cortex is indicated by very mild perivascular cuffing (arrow) and parenchymal inflammatory cells (circle) (D). Numerous CD68-positive macrophages/microglial in the parenchyma and perivascular location (E), and a CD8-positive lymphocyte adjacent to a neuron (F). Viral RNA in the anterior horn cells of the spinal cord (G) and viral antigens in neuronal body and process in the hypothalamus (H). Adjacent sections of the same neuron that was positive for viral RNA (I) and antigens (J). (A–D: hematoxylin and eosin stains; E, F, H, J: immunohistochemistry/peroxidase/DAB; G, I: ISH/nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate stains. Original magnification: (A) 4×; (B, D) 10×; (C, F) 40×; (G, H, I, J) 20×.
included the entire gray matter of the spinal cord from sacral to cervical levels, the entire medulla, pontine tegmentum (sparing anterior pontine nuclei area), tegmentum of the midbrain (sparing the cerebral peduncle but not the substantia nigra), hypothalamus, and subthalamic and dentate nucleus (Fig. 1A, C). Inflammation was also found in the cerebral
There was no noteworthy inflammation within the caudate nucleus, putamen, globus pallidus, thalamus, and mamillary body, although focal areas in the periphery or inferior parts of these structures and other areas adjacent to the hypothalamus occasionally showed mild inflammation. Likewise, very focal areas of white matter adjacent to inflamed anterior horns also showed perivascular cuffing. The olfactory bulb and cerebellar cortex, including Purkinje cells, were generally free of inflammation.

The inflammatory response consisted of perivascular cuffing, parenchymal infiltration by inflammatory cells, and microglial nodules (Fig. 2A). The inflammatory cells usually consisted of macrophage/microglia with lymphocytes, plasma cells, and neutrophils in varying combinations. In some areas, neutrophils were more prominent, but usually, their numbers were insufficient to be characterized as abscesses. Edema was variable, but in the most severely affected areas, the parenchyma seemed microcystic (Fig. 2B). Neuronophagia in various stages was noted, but viral inclusions were absent (Fig. 2C). Mild meningitis was found throughout the CNS, particularly over areas where there was parenchymal inflammation. In the cerebral cortex, inflammation was usually represented by mild perivascular cuffing (often a single layer of inflammatory cells) involving 1 or 2 adjacent vessels and the occasional small microglial nodule (Fig. 2D).

Most parenchymal inflammatory cells were strongly CD68-positive macrophages/microglia (Fig. 2E). CD8-positive lymphocytes were usually absent, but if present, they were found rather sparsely in the perivascular space or parenchyma. Rarely, CD8-positive lymphocytes were adjacent to neurons (Fig. 2F). Inflammation was observed in 1 dorsal root ganglion, but peripheral nerve, intestinal autonomic ganglia, and skeletal muscle had no inflammation. There was also no evidence of inflammation in the other non-CNS organs, including the heart and intestines. Varying degrees of edema were observed in the lung parenchyma, but no convincing inflammation was found.

Detection of Viral Antigens and RNA

Viral antigens were detected to varying degrees in neuronal cell bodies and processes in different parts of the CNS, particularly in the most intensely inflamed areas such as the spinal cord, brainstem, dentate nucleus, and hypothalamus. In the spinal cord, viral antigens were mainly detected in anterior horn cells (Fig. 2G). In general, however, neuronal staining was focal and typically involved only 1 or at most a few adjacent neurons even in the most intensely inflamed areas (Fig. 2G–J). Virus-positive neurons were very rare in the cerebral cortex. Inflammatory phagocytic cells participating in neuronophagia occasionally showed viral antigen in their cytoplasm. No positive staining was found in dorsal root and autonomic ganglia, skeletal muscle, or other non-CNS organs.

In general, ISH using both DNA probes and riboprobes showed findings that closely paralleled IHC findings (Fig. 2I, J). In the positive neurons, staining was confined only to the cytoplasm of the neuronal body and/or neuronal processes, with no nuclear staining in both assays. Staining was often granular in the IHC assay. There was no evidence of virus in the ependyma, astrocytes, oligodendrocytes, choroid plexus, and blood vessels. Overall, there did not seem to be major differences in the sensitivity of the 2 assays of viral detection, and all of the IHC- and ISH-positive and negative controls gave the expected results.

DISCUSSION

The distribution and varying intensity of inflammation in our cases of fatal EV71 encephalomyelitis seem to be distinct and stereotyped, suggesting a similar pattern of viral spread into and within the CNS. As far as we are aware, neuropathologic evidence for this has not been previously reported in EV71 infection. Based on these findings, we speculate that 1 of the major pathways for virus entry into the CNS is via the peripheral nervous system, and from there, virus spreads rostrally up established neural pathways. The most obvious pathway that seems to be involved is the motor pathway. In the spinal cord, all cord levels have more or less the same intensity of inflammation and viral antigens/RNA in the anterior horn cells. In contrast, the degree of inflammation and evidence of virus in the motor cortex was far less. This suggests simultaneous retrograde viral spread up somatic motor nerves to infect anterior horn cells and then up the corticospinal tract to infect neurons in the motor cortex. In this scenario, it is assumed that the first areas (anterior horn cells) to be infected showed the highest degree of inflammation.

The distribution of inflammation in the brainstem was consistent with this mode of spread because motor nuclei were found in all of the areas that had marked inflammation. As in the spinal cord, we postulate that virus may have traveled up cranial nerves with motor components (3rd to 12th nerves) to infect the corresponding motor nuclei in the different parts of the brainstem, and subsequently spread up corticobulbar tracts to the motor cortex. Anterior pontine nuclei (which are not motor nuclei and do not have connections to the ascending corticospinal/corticobulbar tracts) were never found to be inflamed, nor did they show evidence of virus infection.

Further spread within the brainstem via other pathways such as the reticular formation may have occurred as suggested by the presence of inflammation in the central regions of the brainstem. Likewise, hypothalamic involvement can be via its extensive and complex interconnections with the brainstem and spinal cord. Involvement of the dentate nucleus may be through its connections to the inferior olivary nucleus, red nucleus, or other pathways.

Interestingly, the distribution of inflammation in our cases seems to bear a close resemblance to the rare encephalitic form of poliovirus infection (25). It is well known that in poliovirus infection, there is a higher incidence of paralysis after tonsillectomies and intramuscular injections, suggesting a role for viral spread by neural pathways (25). Recent evidence from transgenic mice expressing the human poliovirus receptor had increasingly suggested that poliovirus is able to spread via neural pathways into the CNS (20). Moreover, evidence from both our EV71 mouse model
receptors might account for this observation (27, 28). If such receptors are identified, it is difficult to speculate if differences in viral properties are unknown. Because the receptor for EV71 is different from that of EV70, the disease caused by EV71 was strongly suggested to differ in viral spread occurs by neural pathways into the CNS. Interestingly, in contrast to EV71 encephalomyelitis, the thalamus in polioencephalitis was reported to be severely involved, whereas the inferior olivary nucleus was apparently spared (25). The reasons for these differences are unknown. Because the receptor for EV71 is not the same as the poliovirus receptor and has yet to be identified, it is difficult to speculate if differences in viral receptors might account for this observation (27, 28).

The other possible pathway for viral entry into the CNS would be by hematogenous route and subsequent crossing of the blood-brain barrier. The apparent synchronous nature of anterior horn cell involvement and perhaps the involvement of nonmotor areas such as the hypothalamus and dentate nucleus might support this route of viral spread. If this occurs, certain groups of neurons would necessarily be more susceptible to EV71 infection, thereby resulting in the stereotyped distribution of inflammation. Whether or not this route plays a significant role in EV71 encephalomyelitis and why certain neurons can be more susceptible than others to infection require further investigation.

The peripheral autonomic and sensory nervous system does not seem to be involved in retrograde EV71 viral spread into the CNS. Occasional involvement of the dorsal root ganglion can represent rare anterograde viral spread through its connections to anterior horn cells. It is possible that more widespread centrifugal spread into the peripheral nervous system, as occurs in rabies, might occur if EV71 encephalomyelitis was more chronic.

Detection of viral antigens and genome in the neuronal bodies and processes but not in glial or other cells confirmed that EV71 is neuronotropic. Because infected neurons undergo neuronophagia, direct viral cytolysis is likely an important mechanism of cell damage. Extensive involvement of the medulla, including the nucleus ambiguus, by inflammation, edema, and neuronal loss, was probably sufficient to cause sudden cardiovascular collapse and the presumably neurogenic pulmonary edema in our cases (4, 9). All of our cases succumbed to the disease within 2 to 3 days of illness because most of them presented to the hospital severely ill and deteriorated precipitously. This clinical course would be consistent with a rapid entry of virus into the CNS over a few days.

Brain MRI findings generally seemed to correlate with the distribution of inflammation in our cases, although we are not aware of any reports of MRI-detectable lesions in the cerebral cortex (10, 11). It is probably related to the very focal and mild inflammatory foci found in this location. Absence of virus in non-CNS organs such as the spleen and Peyer patches of the small intestines does not imply that these organs cannot be infected because the tissues obtained from our autopsy cases probably represented a later stage of the disease. If poliovirus infection represents a model for EV71 infection, it should be possible to detect the viral presence in lymphoid organs in the early stage of infection (29), but as far as we are aware, this has not been reported to date.

Few CD8-positive (cytotoxic) lymphocytes were found in areas with abundant neutrophils and a predominance of CD68-positive macrophages/microglia. This finding is consistent with the short duration of the encephalitis and with the assumption that the recruitment of CD8 lymphocytes into the CNS would take longer because it is an adaptive, rather than innate, immune response. Other authors have reported an apparent reduction in CD8- and CD4-positive cells in peripheral leukocytes in patients with EV71 infection and pulmonary edema (30), and it is possible that this may also contribute to the paucity of CD8-positive cells in the CNS. Nonetheless, CD8-positive lymphocytes seem to have a role in the immune response to this infection, and this issue requires further evaluation.

ACKNOWLEDGMENTS

The authors thank Dr. Hiroyuki Shimizu (National Institute of Infectious Disease, Tokyo, Japan) for the antibodies used for immunohistochemistry; and the Ministry of Health, Malaysia, for some of the cases studied.

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