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Laboratory Investigation of a Suspected Enterovirus 71 Outbreak in Central Taiwan

Abstract

Enterovirus 71 (EV71) was first identified in the US in 1969. It was also, of all known enteroviruses, the last one identified. Subsequently, infections have been reported from all over the world. The virus can induce, in addition to some special clinical symptoms such as hand-foot-and-mouth disease（HFMD）and herpangina, serious complications of the nervous system such as meningitis and encephalitis. Its pathogenicity therefore is high. Between January and May 1999, of all suspected EV71 cases reported by contract laboratories in the northern, central, southern, and eastern parts of Taiwan, only three were confirmed. However, since June 1999, one provisional medical center in central Taiwan unusually reported in series six EV71 cases. To avoid any panic of the public resulting from inadequate evaluation, Division of Viral Diseases and Division of Surveillance of Center for Disease Control in Taiwan immediately conducted relevant laboratory and epidemiological investigations of the incident. By visiting cases, understanding the laboratory procedures of the said provisional medical center, and further laboratory testing of specimens, it was decided that the six cases were Coxsackie A16 virus infections.

Introduction

Enteroviruses are micro RNA viruses, including 3 types of polioviruses, 29 Coxsackie viruses of A and B groups, 31 ECHO viruses, and 4 enteroviruses (68-71). They have many serotypes and are ubiquitously found. The mode of

transmission is usually fecal-oral, although they can also be transmitted by droplets and secretions of infected persons. Currently, with the exception of polioviruses, no vaccines are available for prevention, nor are medicines for treatment. The only prevention against infection is by maintaining sound personal hygiene. There was a serious outbreak of enterovirus infection in Taiwan in 1998. Of the 78 deaths, by laboratory testing, no viruses were isolated in 31 cases, 9 cases were not tested, and the remaining 34 cases were found to be enterovirus 71 positive. EV71 was therefore considered the major cause of the serious complications. This year, under the optimum efforts of the health care agencies at all levels, enterovirus infection has been brought under control. By May 1999, of all EV71 cases reported by contract laboratories, only three were confirmed. When six confirmed EV71 cases were reported one by one from central Taiwan, and mostly by a clinic in Miaoli County, and yet no major outbreak of any infection was reported from that area at that time, a further investigation was called for to verify the laboratory testing.

Materials and Method

1.Medical record and case interview

Information was collected to understand the backgrounds of the cases.

2.Visit to the reporting clinic

A visit was made to the reporting clinic to understand the process of specimen collection and to investigate the possibility of contamination.

3.Visit to the laboratory of the provisional medical center

A visit was made to the laboratory of the provisional medical center to ascertain if the laboratory procedures were adequate, the facilities were appropriate, the cells were properly maintained, and the reagents used were correct; and whether there was any chances of contamination during the course of testing.

4.Specimen collection and laboratory testing

Viral culturing: throat swabs collected by the clinic and virus strains cultured by the provisional medical center were brought back for further testing by Division of Viral Diseases of Center for Disease Control in Taiwan. Specimens of the throat swabs were inoculated on Hep-2 and RD cell strains, placed in a CO_2 incubator for culturing at 36^0 C, and observed and recorded everyday. If CPE emerged, the specimen was identified with immunofluorescence assay(IFA) and neutralization test. RNA was extracted from the virus strains for polymerase chain reaction (RT-PCR) and genetic

sequencing. Neutralization test was also conducted on sera of patients and their virus strains.

Antibodies: The local health bureau was asked to collect sera of patients for antibody neutralization test. Due to refusal of testing by the patient, no sera was collected from Case No.1. Sera specimens were first diluted at 1:8, heated at 56^0 C for 30 minutes, and placed in 96 well microplate for further dilution. EV71 was then added into the microplate and incubated in a CO_2 incubator at 36^0 C, observed and recorded everyday for CPE to determine the antibody titer.

Results

1.Case interview: findings are shown in Table 1.

2.Visit to the reporting clinic: nurses in the Pediatrics department took rectal and throat swabs, serum, and spinal cord fluid from Case No.1; physicians took throat swabs from cases 2-5.

3.Visit to the laboratory of the provisional medical center: the facilities and quality of manpower were quite adequate. A careful investigation of the testing procedures, however, revealed that the testing for IFA was questionable. The test kits used by the center were not adequate; the possibility of cross-reaction between enteroviruses of different serotypes could not be ruled out. This would have an effect on the correct reading of the virus serotypes.

4.Laboratory testing (see Table 2)

Virus culturing: findings through virus isolation (IFA testing), RT-PCR, and genetic sequencing confirmed that all six cases were Coxsackie A16 infections. Results of the antigen neutralization test also indicated that Coxsackie A16, and not EV71, was responsible for the infections.

Antibody testing: by antibody neutralization test, it was found that blood of all cases, with the exception of Case No.5, contained both EV71 and Coxsackie A16 antibodies.

Discussion

Enteroviruses are distributed widely and have survived in the environment for centuries. They come in many serotypes, and cause many different diseases. Although many of those infections are either asymptomatic or exhibit mild common-cold-like symptoms, when the virus attacks the central nervous system relatively serious complications may develop. In April 1998 in Taiwan, A sudden increase of the HFMD reported by physicians of the sentinel reporting system was noted. In May and June, the number of reported cases reached a climax. Throughout the year, 405 cases, mostly young children with a high fatality rate, were reported as suspected enterovirus infections with serious complications. EV71 was suspected as the likely major cause of the serious

complications. The presence of EV71 was an important index of serious enterovirus infections.

The six suspected cases of EV71 infections reported in series by a provisional medical center in central Taiwan, were characterized by HFMD symptomatology, and all cases were under five years of age. As interviews of cases did not reveal any serious complications, the incident was initially considered a Coxsackie A16 infection, as that virus could also induce HFMD, with, however, more moderate symptoms. By IFA and RT-PCR alone, because of cross-reaction, the differences between EV71 and Coxsackie A16 are often misleading. To avoid confusion, a further test for nucleic acid sequencing is necessary.

In addition to the virus isolation of specimens collected by the initial clinic from patients, the Division of Viral Diseases of Center for Disease Control in Taiwan had also asked the local health bureau to collect sera from patients for testing of antibodies. The presence of antibodies should indicate infection, and eliminate the chances of contamination of specimens. Findings from virus isolation and IFA all indicated Coxsackie A16 infection. Results from antigen neutralization test of the sera and virus strains of the patients also indicated that the six patients were infected by Coxsackie A16. RT-PCR and genetic sequencing, after being sent to the US CDC for matching, also proved to be Coxsackie A16. These laboratory findings did not correspond with findings of the provisional medical center. Observation of the laboratory procedures of the provisional medical center revealed, however, that the process of IFA was questionable. Due to reason of cross-reaction between Coxsackie A16 and EV71, reagent 3323 was unable to differentiate the two viruses. A further use of EV71-specific reagent 3324 was essential for the differentiation. For the testing of antibodies, with the exception of Case No.1, from whom no sera specimens were collected, Coxsackie A16 antibodies were found in all patients, indicating that they had been infected by this agent. As no paired sera were collected, this finding alone could not be used for diagnosis. The EV71 antibodies found in cases 2, 3, 4, and 6 could have been elicited from the outbreak of last year. That the titer of the EV71 antibodies in cases 2, 4, and 6 was higher than that of Coxsackie A16 could be because the present Coxsackie A16 infection had reinforced the already existing EV71 antibodies.

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Table 1. Backgrounds of Suspected EV-71 Patients, Changhua and Miaoli Counties

Case No.	Birth Date	Date of Onset	Specimens Collected from	County	Date Reported
1	05/03/ 97	06/05/99	A Pediatrics	Changhua	06/22/99
2	04/07/98	07/05/99	B Pediatrics	Miaoli	07/19/99
3	09/03/96	07/27/99	B Pediatrics	Miaoli	08/05/99
4	03/31/95	07/26/99	B Pediatrics	Miaoli	08/05/99
5	03/06/97	07/27/99	B Pediatrics	Miaoli	08/05/99
6	12/03/94	08/01/99	B Pediatrics	Miaoli	08/13/99

Table 2. Laboratory Findings

Case No	Virus Isolation				Serum Antibody titer				RT-PCR and Genetic Sequencing
	Type	Identification			EV-71	CA-16			
		FA	Neutralization test			Prototype	E98974[a]	E990128[b]	
			EV-71 Antiserum	CA-16 Antiserum					
1	CA-16	+	–	+	ND[c]	ND	ND	ND	CA-16
2	CA-16	+	–	+	≧1024	256	64	64	CA-16
3	CA-16	+	_	+	8	128	8	8	CA-16
4	CA-16	+	_	+	≧1024	256	64	32	CA-16
5	CA-16	+	_	+	<8	256	32	32	CA-16
6	CA-16	+	_	+	512	128	8	16	CA-16

a: CA-16 virus strain isolated from patient in Hsiluo area, Yunlin County, on October 22, 1998.

b. virus strain isolated from Case No. 3

c. not tested.