ISSN 1021-366X

External Quality Assessment of Enterovirus Testing of Laboratories Contracted for the Testing of Viral Infections

Abstract

To assess the accuracy in the isolation and differentiation of enteroviruses of laboratories under contract, the Center for Disease Control conducted, in early March 2001 before the expected attack of the enterovirus epidemic, proficiency testing of 11 laboratories under contract to correctly understand the trend of enterovirus infection, and to upgrade the quality of overall disease control. Each laboratory was supplied with nine specimens. Upon receipt of the specimens, the laboratories were told to isolate viruses with cell strains routinely in use. Positive specimens were then assessed by immunofluorescent assay. Laboratories could, on their own decision, use neutralization test, PCR and gene sequencing for further confirmation. However, typing by fluorescence was used as a basis for the assessment.

The results were, two laboratories had full marks; whereas one failed at a score of 50. The average score was 81. Major errors were in virus assessment. Of a total of 99 specimens, viruses were correctly isolated in

96 (96.9%), and the rest three were negative. Of them, 78 (81%, 78/96) were correctly assessed. The major problem in the assessment was noted in specimens mixed with two types of enteroviruses (poliovirus II and poliovirus III). Only three in the 11 laboratories made correct assessment; the rest eight succeeded in assessing only one type of viruses. Three laboratories did not carry fluorescent reagents for CA9, their reading was Pan-Ev. One carried overdue reagents.

Of the 11 laboratories, nine kept complete observation records of virus isolation of each cell strain and specimen, and tabulated their data. In all cell strains, monkey kidney cell line (MK2, Vero, GMK) had the highest isolation rate of 77.5%; human heteroploid cell (HEP-2, A549, HeLa, RD) and human diploid bifroblast (Hel, MRC-5) came the next at 72.0% and 68.8% respectively. By individual cell strain, RD had the highest virus isolation rate of 95.8%.

Key words: enterovirus, proficiency testing, neutralization test, sequencing, PCR

Introduction

In March 1999, the Center for Disease Control contracted several laboratories in the northern, central, southern and eastern parts of Taiwan for the surveillance of enterovirus to understand the trend of infection and also changes in virus types. Specimens came from two sources, reported cases of enterovirus infection with serious complications, and cases of hand-foot-mouth disease or herpangina referred for testing by sentinel physicians. Since 2000, 11 laboratories of the National Taiwan University Hospital, Tri-Service General Hospital, Chang Gung Linkou Hospital, Taichung Veterans General Hospital,

China Medical College Hospital, Changhua Christian Hospital, Cheng Kung University Hospital, Kaohsiung Medical College Hospital, Kaohsiung Veterans General Hospital, Chang Gung Kaohsiung Hospital, and Tzuchi Hospital have participated in the program. To assess these laboratories the accuracy in the isolation and differentiation of enteroviruses, and to upgrade their testing capabilities for enteroviruses[5], a proficiency testing of these laboratories was conducted sometime before the expected attack of the enterovirus infection. Each laboratory was supplied with nine specimens. They were told to isolate viruses with cell strains routinely in use. Positive specimens were then assessed with immunofluorescent assay. They could, on their own decision, use neutralization test and PCR[6] for further confirmation. However, typing by fluorescence was used as a basis for the assessment.

Materials and Methods

Time of Assessment

Laboratories were notified of the time of assessment by official letters. Specimens were sent by express mail the first thing on 18 March 2002. The laboratories were reminded again by e-mail to make sure that specimens were collected on the same day. They were told to fax within 17 days upon receipt of specimens (by 5 April) findings and testing records to the Center for Disease Control. The Laboratory of the Center conducted at the same time same testing to understand the adequacy of the specimens prepared for the proficiency testing.

Specimens for Proficiency Testing

Of the nine specimens, seven contained one type of virus, two had two types of viruses, and one was free of virus. Viruses were suspended in DMEM-2%FBS solution to maintain the stability of specimens. The titer

was between 10 to 100 $TCID_{50}$/0.05ml. Standard viruses bought from ATCC (poliovirus 2, 3; CB4; E6) and indigenously isolated strains (EV71; CA16; CA9; CB3; CA4) were used for the preparation of the specimens (Table 1)[(3)]. Specimens were randomly coded to prevent laboratories from sharing information.

In the preparation of specimens, viruses were first tested for their titer, adjusted concentration to 10 $TCID_{50}$/0/05ml and CA4 to 100 $TCID_{50}$/0.05ml. To assure the uniformity and stability of specimens, each type of virus was mixed evenly by vortex before packing, 1ml for each tube. After preparation, one tube was taken from each specimen for quantitative analysis to assure the concentration of viruses. Blind codes were labeled on tubes for each laboratory, and kept temporarily in freezer at –80℃. Specimens were coded as follows:

Virus specimens: specimen 1: EV-71 specimen 2: CA16 specimen 3: CA9 specimen 4: CB3 specimen 5: CB4 specimen 6: blank specimen 7: CA4 specimen 8: E6 specimen 9: P2 + P3

Cell Line and Anti-Sera

By inquiries, it was noted that the cell strains and anti-sera the laboratories were using were: two laboratories had their own anti-sera pooled from anti-sera products bought from ATCC (American Type Culture Collection) (Laboratories Nos. 7 and 8); and anti-sera products bought from Denka of Japan (Laboratories Nos. 1 and 9). Cell line were either bought from ATCC, from the Bacteriological Center of the Hisnchu Food Industry Research and Development Institute, or self-prepared Hel cell strains. Cell stains of Laboratory No. 1 were MK2, RD, Hel, Vero, A549 and HEP-2; of Laboratory No. 2 were MK2, RD, A549, MRC-5; of Laboratory No. 3

were RD, Vero, HEP-2, MRC-5; of Laboratory No. 4 were RD, Vero, A549, HEP-2; of Laboratory No. 5 were RD, Vero, A549; of Laboratory No. 6 were RD, Vero, HEP-2, MRC-5; of Laboratory No. 7 were RD, A549, MRC-5, GMK; of Laboratory No. 8 were RD, Hel, A549, GMK, Fibro; of Laboratory No. 9 were MK2, RD, Hel, A549; of Laboratory No. 10 were MK2, RD, Hel, A549, HEP-2, MRC-5; and of Laboratory No. 11 were RD, HEP-2.

RT-PCR Reactions

The primers used were synthesized by each laboratory from research findings[9,10,11] within the VP1 and 5 ranges.

EV2 – TCCGGCCCCTGAATGCGGCTAATCC (446-470)
EV1 – ACACGGACACCCAAAGTAGTCGGTCC (559-533)[9]
011 – GCICCIGAYTGITGICCRAA (3408-3389)
012 – ATGTAYGTICCICCIGGIGG (2951-2970)
040 – ATGTAYRTICCIMCIGGIGC (2951-2970)[10]
EV1 – CAAGCACTTCTGTTTCCCCGG (164-184)
EV2 – ATTGTCAACCATAAGCAGCCA (599-578)[11]

Results

The requirement was that each of the 11 laboratories used the cell strains routinely in use for the isolation of viruses, and assessed positive specimens by the immunofluorescent assay. They could decide whether to further confirm the findings by neutralization test and PCR methods.

Cell Line, Anti-Sera and Reagents Used and Methods of Identification

Cell line routinely in use were used for the isolation of viruses. The cells, anti-sera and reagents used and identification methods are shown in Table 2. Each laboratory used more than two cell strains to isolate enterovirus. Of

them, 11 used RD cells, eight used A549, six used HEP-2, five used MRC-5 and Vero, and four used MK2.

Fluorescent reagent of the Chemicon was used by all. Two laboratories (Nos. 1 and 7) partially used reagent for research purpose by diluting it. The rest laboratories used diagnostic reagent. One laboratory (No. 3) used overdue fluorescent reagent by mistake. Three laboratories (Nos. 6, 8 and 10), for rarely isolating CA9 virus, did not carry fluorescent reagent, their CA9 could only isolate enterovirus. Eight laboratories (Nos. 2, 3, 4, 5, 7, 8, 9 and 10) detected only one type of virus in specimen 9 (containing P2 and P3 mixed viruses).

Of the four laboratories (Nos. 1, 7, 8 and 9) capable of performing neutralization test, two (Nos. 1 and 9) bought pooled anti-sera from Denka; the other two (Nos. 7 and 8) home-made their own pooled anti-sera from the anti-sera bought from ATCC.

Three laboratories (Nos. 5, 7 and 8) used PCR after isolation of Pan Ev. Laboratory 5, in the CA4 virus isolation, found it negative but Ev when 5'NCR region primer was used for RT-PCR. Each laboratory could perform virus isolation and fluorescent test, and it was also a major point of assessment. The laboratory was scored wrong on this point. Laboratory 7 succeeded in assessing CA4 virus by using VP1 region primer for RT-PCR and sequencing. Laboratory 8, for short of fluorescent reagent for CA9, used VP1 region primer for the RT-PCR of E30 and EV-71-specific to find them negative, and CA9 virus assessment as Pan Ev. Laboratory 2 was overdue in submitting report.

Virus Isolation and Identification

In all 99 specimens, viruses were isolated in 96 of them (96.9%), and three were false negative. Of the 96 virus strains isolated, 78 were correct (81%, 78/96). Virus isolation and assessment by laboratory is shown in Tables 2 and 3. Laboratories 4 and 5 found negative in the isolation of EV-71, E6 and CA4, perhaps due to the decline in the sensitivity of cells. They should strengthen quality control of cells by sensitivity testing of cells or micoplasma test.

Fluorescent reagents manufactured by Chemicon are for both research and diagnosis purposes. The reagent for research purpose should be diluted before use. In the supervision of laboratories for improvement, it was found that the CB blend for research use was already diluted, and it was not noted on this testing that the reagent was already ineffective. Quality control therefore is most essential. Eight laboratories (Nos. 2, 3, 4, 5, 7, 8, 9 and 10) detected only one type of virus in specimen 9 containing mixed P2 sand P3 viruses.

The pooled sera home-made from anti-sera bought from ATCC was adjusted for the ATCC titer while in preparation. Misjudgment likely to occur by heterotiters of viruses was not tested.

Cell Susceptibility

Cell strains used for the isolation of viruses in the present proficiency testing were varied. Nine of the 11 laboratories kept complete records of virus isolation for each cell strain and specimen. Three types of cell strains were used, monkey kidney cell lines, human diploid fibroblasts, and human heteroploid cells. Monkey kidney cell lines had the highest isolation rate

(n=10) of 77.5%; and the human heteroploid cells (n=7) and human diploid fibroblasts (n=21) came the next at 72.0% and 64.3% respectively (Tables 4 and 5).

Of the enterovirus specimens, CB4, CA9 and CB3 had high isolation rates of 86.8%, 81.6% and 81.6% respectively. CA4 had the lowest isolation rate of 31.6%. By individual cell, RD cell had the highest isolation rate of 95.5%; and GMK and Vero came the next at 87.5% and 80.0% respectively.

Statistical Methods and Assessment of Results

The total score of the proficiency testing was 10 as specimen 9 contained two types of viruses. This was a qualitative testing, and a correct detecting of the viruses in the specimens was rated correct, and percentage was calculated. Of all, two laboratories had full marks; two had a score of 90; four had 80; two had 70; and one had 50, averaging 81 (Table 6). All laboratories were notified by official letters of the test results in codes.

Improvement of Laboratories Not Performing Well in the Proficiency Testing

Discussions were held with the technicians in charge of the laboratories concerned by telephone. They were asked to repeat the testing until the findings were correct. They were also told to improve and submit records for follow-up by specialists. They could come to the Laboratory of the Center for on-job training.

Discussion

Taiwan is in the sub-tropical zone. Enterovirus infection prevails in May through October each year. There are some sixty already known types of enterovirus, inducing different clinical symptoms such as hand-foot-mouth

disease and herpangina. Serious complications include acute myocarditis, pericarditis, aseptic meningitis, and encephalitis. Except a few serotypes that induce serious clinical symptoms, most infections are insignificant. It is, therefore, difficult to decide from clinical symptoms whether it is enterovirus infection, laboratory testing is necessary. However, there has not yet been any cell that can culture all types of enterovirus. Some serotypes may require newborn sucking mice for virus isolation. Animal experiment is always time-consuming. Each laboratory therefore uses cells for virus isolation as a standard procedure. They use more cells to improve isolation rates. Virus isolation by laboratories not only affects the diagnosis and treatment of physicians, it has impact on disease control measures. The present testing focused on the capabilities of virus isolation.

There are several factors that may affect virus isolation, the cell strains used, the sera placed in the culture media, temperature, amount of viruses inoculated on specimens, and the cold chain of specimen transportation, for instance. The specimens used in the present testing were carried in low-temperature containers specifically for the transportation of specimens, and delivered by express mail all at the same time. The question of differences in cold chain process could be eliminated.

Cell strains of different sources show different susceptibility to viruses. Even cell strains of the same source, after keeping for some time in the laboratory, their younger generations may show different susceptibility to viruses. During the cohort culturing of cell strains, they can be contaminated by micoplasma, their susceptibility to viruses may decline. To maintain sound susceptibility of cell strains to viruses, laboratory technicians must regularly

check if there are micoplasma infections and also susceptibility to viruses.

The fetal bovine sera in the culture media is also a factor of virus isolation. The small amount of hormone, such as cortison, or unknown inhibitors, contained in the fetal bovine sera of different batches may also affect the virus isolation of certain serotypes. A correct way is to pretest the fetal bovine sera to be used for the culturing of cells to see if they are inhibitive to the viruses to be isolated.

The volume of sample inoculation into appropriate cell line has great effect on the virus recovery rate. Virus recovery rate will not be affected even the inoculated volume is reduced by half if the specimen has already contained in the specimens is at its low limit for virus recovery, it will be difficult to isolate the virus from half inoculated volume than usual volume. It is better to take fixed and appropriate volume of samples for virus isolation. The laboratory staffs have similar experience during the AFP surveillance for poliovirus identification. Usually, after inoculation, only one of two cultured tubes display CPE. On visits to laboratories, it was found that the amount of viruses inoculated in specimens was not constant. To inoculate more cell strains, the number of cell tubes for inoculation was reduced by half, from the regular two tubes to one. Whether isolation rates could be improved by this way remains to be investigated and discussed.

Methods for the assessment of enterovirus include serum neutralization test[7], fluorescent antibody method, ELISA, hibridizatin, PCR[7,8], and nucleic acid sequencing. For reasons of manpower and technologies of the laboratories under contract, all assessment methods, with the exception of the fluorescent antibody method, are not yet included in the testing. More should be done in

the future. Fluorescent assay is rapid and convenient, and is commonly practiced by clinical laboratories. However, the method has a significant individual difference, and therefore, requires experienced technicians for close observation. If there are two types of viruses in an infected cell, unless closely observed, one of types of virus could easily be overlooked. The present testing verified this fact.

The present proficiency testing of the enterovirus testing of the laboratories under contract followed the model for poliovirus adopted by member states of the World Health Organization. They use specimens containing one or two mixed types of poliovirus, fecal specimens of enterovirus, and also specimens free of viruses, for isolation and typing[(4)]. A score of 80 is qualified. In the present testing, eight laboratories each had a score of more than 80.

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Table 1 Amount of viruses Contained in Specimens for Enterovirus Testing by Contracted Laboratories

Specimen	Viruses		
	Serotype[a]	Virus Strain	$TCID_{50}$/0.1ml
1	EV71	E010234	12
2	CA16	E010249	64
3	CA9	E010277	64
4	CB3	E980646	50
5	CB4	J.V.B	64
6	Neg.	blank	-
7	CA4	E980273	2000
8	E6	D'Amori	100
9	P2+P3		38
		sabin strain	74

Note:[a]CA=Coxsackievirus A; CB=Coxsackievirus B; Ev=Enterovirus; P=Poliovirus

Table 2 Cells, Reagents and Assessment Methods Used by Contracted Laboratories for the Proficiency Testing

Lab Code	Cell Strains	Assessment Methods			Result（correct/specimen）			
		IF	NT	PCR	Isolation	IF	NT	PCR
1	MK2,RD,Hel,Vero,A549, HEP-2	A	C	-	9/9	10/10	1/1	-
2	MK2,RD, A549,MRC-5	B	-	-	9/9	8/10	-	-
3	RD, Vero, HEP-2,MRC-5	A	-	-	9/9	5/10	-	-
4	RD, Vero,A549, HEP-2	A	-	-	8/9	8/10	-	-
5	RD, Vero,A549	A	-	5'NCR	7/9	7/10	-	1/1
6	RD, Vero, HEP-2,MRC-5	A	-	-	9/9	8/10	-	-
7	RD, A549,MRC-5,GMK	B	H	VP1	9/9	9/10	4/4	1/1
8	RD, Hel, A549,GMK,Fibro	A	H	VP1	9/9	7/10	3/4	2/2
9	MK2,RD,Hel, A549	A	C	-	9/9	9/10	2/2	-
10	MK2,RD,Hel,549, HEP-2,MRC-5	A	-	-	9/9	8/10	-	-
11	RD,HEP-2	A	-	-	9/9	10/10	-	-

Notes: IF=Immunofluorescent method; NT=Neutralization Test

1.All laboratories used products of Chemicon for immmunofluorescent method; A for diagnostic, and B for research purposes.

2.Reagents used in neutralization test: H=home-made pooled anti-sera; C=products of Denka

3.Laboratories 6, 8 and 10 did not carry CA9 fluorescent reagents.

4.Laboratory 3 used overdue reagent.

5.Laboratories 2, 3, 4, 5, 7, 8, 9 and 10 isolated only one type of viruses in specimen 9 (containing P2 and P3 mixed viruses).

6.Laboratory 2 late in submitting report.

Table 3 Results of Proficiency Testing for Enterovirus by Laboratory

Lab code	Specimen										
	1	2	3	4	5	6	7	8	9	Isolation	Assessment
	EV71	CA16	CA9	CB3	CB4	（-）	CA4	E6	P2+P3	（n=9）	（n=10）
1	EV71	CA16	CA9	CB3	CB4	（-）	Ev	E6	P2+P3	9	10
2	EV71	CA16	CA9	CB3	E4	（-）	Ev	E6	P2	9	8
3	EV71	CA16	E4	E4	E4	（-）	E4	E6	P2	9	5
4	-	CA16	CA9	CB3	CB4	（-）	Ev	E6	P3	8	8
5	EV71	CA16	CA9	CB3	CB4	（-）	-(*）	-	P3	7	7
6	EV71	CA16	Ev	CB3	Ev	（-）	Ev	E6	P2+P3	9	8
7	EV71	CA16	CA9	CB3	CB4	（-）	CA4	E6	P3	9	9
8	EV71	CA16	Ev	CB3	CB4	（-）	CB4	E6	P3	9	7
9	EV71	CA16	CA9	CB3	CB4	（-）	Ev	E6	P3	9	9
10	EV71	CA16	Ev	CB3	CB4	（-）	Ev	E6	P2	9	8
11	EV71	CA16	CA9	CB3	CB4	（-）	Ev	E6	P2+P3	9	10
Total number of specimens: 99											
Isolation	10	11	11	11	11	11	10	10	11	96	
Assess ment	10	11	7	10	8	11	8	10		78	89

Notes: 1. CA=Coxsackievirues A, CB=Coxsackievirus B, Ev=Enterovirus, P=Poliovirus, (-)= virus isolation negative

2. *Virus not isolated but PCR assessment.

Table 4 Isolation Rates by Cell Strains

Specimen	Virus	Monkey kidney cell lines (MK2,Vero, GMK) (n=10)	Human diploid Fibroblasts (Hel,MRC-5) (n=7)	Human heteroploid cells (HEP-2,A549, Hela,RD) (n=21)	Total	%
1	Ev71	8/10	7/7	10/21	25/38	65.6
2	CA16	9/10	7/7	10/21	26/38	65.6
3	CA9	8/10	7/7	17/21	32/38	81.6
4	CB3	9/10	1/7	21/21	31/38	81.6
5	CB4	10/10	2/7	21/21	33/38	86.8
7	CA4	2/10	1/7	9/21	12/38	31.6
8	E6	8/10	7/7	15/21	30/38	78.9
9	P2+P3	8/10	4/7	18/21	30/38	78.9
Total		62/80(77.5%)	36/56(64.3%)	121/168(72.0%)		

Table 5 Isolation Rates by Cell Strains

Specimen	Virus	MK2	Vero	GMK	Hel	MRC-5	HEP-2	A549	Hela	RD
		(n=3)	(n=5)	(n=2)	(n=3)	(n=4)	(n=5)	(n=6)	(n=1)	(n=9)
1	Ev71	2/3	4/5	2/2	3/3	4/4	2/5	1/6	0/1	7/9
2	CA16	2/3	5/5	2/2	3/3	3/4	2/5	0/6	0/1	8/9
3	CA9	2/3	4/5	2/2	3/3	3/4	3/5	4/6	1/1	9/9
4	CB3	2/3	5/5	2/2	0/3	1/4	5/5	6/6	1/1	9/9
5	CB4	3/3	5/5	2/2	0/3	2/4	5/5	5/6	1/1	9/9
7	CA4	0/3	1/5	1/2	1/3	0/4	0/5	0/6	0/1	9/9
8	E6	2/3	4/5	2/2	3/3	4/4	2/5	3/6	1/1	9/9
9	P2+P3	3/3	4/5	1/2	2/3	2/4	4/5	4/6	1/1	9/9
Total		16/24	32/40	14/16	15/32	19/32	23/40	23/48	5/8	69/72
%		66.7	80.0	87.5	46.9	59.4	57.5	47.9	62.5	95.5

Table 6 Testing Results by the Laboratories

Lab Code	1	2	3	4	5	6
Score (%)	100	80	50	80	70	80
Lab Code	7	8	9	10	11	
Score (%)	90	70	90	80	100	