Original Report

Enterovirus isolation from children with acute respiratory infections and presumptive identification by a modified microplate method

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Objective: To evaluate a modified microplate method, utilizing HEF, HEp-2, Vero, MDCK and newly introduced RD-18S and GMK cell lines, for virus isolation.

Methods: From June to October 2001, 723 throat swab specimens taken from children with acute respiratory infections (ARIs) were inoculated onto these cells. To analyze cell sensitivity, we also inoculated 20 serotypes of stocked enteroviruses.

Results: During the period, we isolated 40 Coxsackie A2 (CoxA2), 13 CoxA4, 16 CoxA16, 1 CoxB2, 11 CoxB3, 2 CoxB5, 54 echo16, 2 entero71 and 1 polio2. By observing a cell sensitivity pattern with HEF, HEp-2, Vero, RD-18S, and GMK, we could finally differentiate five enterovirus groups: CoxA except for CoxA16, CoxA16/entero71, CoxB, echovirus, and poliovirus.

Conclusions: With this system, the RD-18S cell line enabled us to isolate CoxA virus, except for CoxA16, for the first time. Differentiation of five enterovirus groups by cell sensitivity simplified the specific identification by neutralization test as a presumptive identification. A modified microplate method may be an appropriate cell combination for virus isolation, especially for enteroviruses, and is expected to be used routinely for virologic diagnosis and to clarify the epidemiology of ARI in children.

Int J Infect Dis 2003; 7: 138–142

INTRODUCTION

Human enteroviruses belong to the family Picornaviridae, and include 64 serotypes, parechovirus 1 (echo22) and parechovirus 2 (echo23).[1] Although most enteroviral infections are asymptomatic, they can cause mild diseases such as respiratory illness, herpangina, hand, foot and mouth disease, and exanthema, mainly during the summer season.[2] These viruses are also associated with aseptic meningitis and encephalitis as central nervous system infections.[2]

Virus isolation is still a sensitive and important method for virologic diagnosis, and is regarded as a 'gold standard'.[3] No one cell line is able to support the growth of all cultivable enteroviruses.[1] It is therefore recommended that cell lines of monkey and of human origin, and of newborn mice, should be used to recover all kinds of enteroviruses.[2] As an alternative, virus isolation using a combination of several cell lines has been attempted.[4–14] Rhabdomyosarcoma (RD), rhesus and/or cynomolgus monkey kidney (MK), human embryonic lung fibroblast (HEF) as diploid cell, buffalo green monkey kidney (BGM) and HEp-2 have been commonly used. It is also suggested that a sensitivity pattern of several cell lines can be used for the presumptive identification of enteroviruses.[14,15]

In the last two decades, we have routinely used the original microplate method (HHVM plate), including HEF, HEp-2, Vero and MDCK cell lines, for virus isolation to clarify the epidemiology of acute respiratory infection (ARIs) in children.[15] This system can isolate not only enteroviruses but also other respiratory viruses. Recently, we tried to use RD-18S and green monkey kidney cells (GMK) to isolate enteroviruses more efficiently than with the original HHVM plate. With this new system, we succeeded further in isolating Coxsackie A (CoxA) viruses other than CoxA16 and in making a presumptive identification for five enterovirus groups.

PATIENTS AND METHODS

Clinical specimens

Between June and October 2001, 723 throat swab specimens were collected from patients with ARIs at pediatric clinics, which collaborate with Yamagata Prefecture in the national surveillance of viral diseases in Japan. Patients were under 15 years old, and were clinically diagnosed as having ARI with fever and/or

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Corresponding Editor: Jane Zuckerman, London, UK

cough and/or rhinorrhea. Six hundred and ten patients (84.3%) were diagnosed as having upper respiratory infections, and 24 (3.3%), 18 (2.5%), 13 (1.8%), 12 (1.8%) and 33 (4.6%) as having lower respiratory infections such as bronchitis, hand, foot and mouth disease, central nervous system diseases such as aseptic meningitis, acute gastroenteritis, and others such as measles, respectively. Patients visited outpatient clinics, and there were no immunosuppressed cases. Throat swab specimens were collected and placed immediately in tubes containing 3 mL of transport medium, including Eagle's minimum essential medium (MEM: Nissui Pharmaceutical Co. Ltd, Tokyo, Japan), penicillin G (50 U/mL, Asahi Chemical Industry Co. Ltd, Toyama, Japan), and streptomycin (0.4 mg/mL, Meiji Seika Kaisha, Ltd, Tokyo, Japan). Specimens were placed in a cooler box and transported for virus isolation to the Department of Microbiology, Yamagata Prefectural Institute of Public Health.

Virus isolation and identification

Virus isolation was performed using a modified microplate method; that is, we added RD-18S and GMK cells to the original four cell lines (HHVMRG plate). We used 96-well tissue culture plates (Greiner, Frickenhausen, Germany) vertically, and prepared two rows of each cell line. HEF, which was originally established by I. Ishiwata, was purchased from Riken Cell Bank, Tsukuba, Japan. HEp-2, Vero and MDCK cells were obtained from Sendai National Hospital, Virus Research Center, Sendai, Japan. The growth medium for RD-18S consisted of MEM with 10% fetal bovine serum (FBS). The growth medium was MEM with 5% FBS and 5% calf serum (CS) for GMK. The maintenance medium was MEM with 2% FBS for both.

Specimens were centrifuged at 3000 rev/min for 15 min. Seventy-five microliters of supernatant were inoculated onto two wells of each cell line. Microplates were centrifuged for 20 min at 2000 rev/min, incubated at 33°C in a 5% CO_2 incubator, and assessed for cytopathic effects (CPEs) up to the 10th day without medium exchange. When a typical CPE of enterovirus was observed, viral fluid was passed to another identical cell line, and then viral identification was carried out.

We made a specific identification of isolates with a neutralization test. We used echovirus pool antisera 'EP 95', and sera anti-CoxA2–6, 8 10, provided by the National Institute of Infectious Diseases, Tokyo, Japan. We also purchased serotype-specific antisera against echovirus and CoxB viruses from Denka, Seiken, Tokyo, Japan. Sera anti-CoxA16 and anti-entero71 were provided by Sendai National Hospital Virus Research Center.

Cell sensitivity test for enteroviruses

After a passage, viral fluid was again inoculated onto the microplate (25 μL/well), as a cell sensitivity test. The microplate included HEF, HEp-2, Vero, RD-18S and GMK cells (HHVRG). To analyze more enterovirus isolates than in the 2001 season, we used enteroviruses stored at −80°C between 1993 and 2000, and inoculated them into the above five cell lines. Finally, we analyzed 20 serotypes and used 1–3 strains for each serotype. We were able to try cell sensitivity tests for different years for CoxA16, CoxB3, CoxB5, echo30, entero71, and polio2.

RESULTS

Number of virus isolates among children with ARIs in Yamagata in the 2001 season

In total, 147 enteroviruses were isolated between June and October 2001 in Yamagata, Japan (Table 1). Echo16 was the most common (54 isolates). This was followed by CoxA2, CoxA16, CoxA4, CoxB3, CoxB5, entero71, and CoxB2, in that order. One polio2 strain was isolated from a child at 6 months of age, who had received oral polio vaccine. We were not able to identify seven isolates that showed enterovirus-like CPEs in HEF and/or in RD18-S. Although we succeeded in amplifying viral genomes by the reverse transcription polymerase chain reaction (RT-PCR) according to Robart's method,[16] we have not tried a sequence analysis for identification.

Echo16 strains were isolated primarily in RD-18S (52/54) and HEF (49/54). Four isolates of echo16 propagated only in RD-18S, and one in HEF. CoxA16 strains were mainly replicated in GMK (15/16) and HEF (12/16). Four isolates of CoxA16 only showed CPEs in GMK. CoxB2, B3 and B5 were all isolated in HEp-2, and some were also propagable in Vero and other lines.

Apart from enteroviruses, 21 adenoviruses, 18 cytomegaloviruses, 14 herpesviruses, 5 rhinoviruses and 1 mumps virus were isolated in the HHVMRG plate during the study period. There were no mixed infection cases.

Cell sensitivity of enterovirus isolates

The results of cell sensitivity tests of enteroviruses after a passage are shown in Table 2. CoxA2, 4 and 10 showed

Table 1. Number of primary enterovirus isolates in five cell lines from children with acute respiratory infections between June and October 2001, in Yamagata, Japan

Serotype	*HEF*	*HEp-2*	*Vero*	*RD-18S*	*GMK*	*Total*
CoxA2				40		40
CoxA4				13		13
CoxA16	12		4	6	15	16
CoxB2		1			1	1
CoxB3		11	4	1	3	11
CoxB5		2	2		1	2
Echo16	49			52		54
Entero71					2	2
Polio2	1	1	1	1	1	1
Not identified	3			5		7

CPEs only in RD-18S. CoxB2, B3, B4 and B5 propagated well in HEp-2, Vero, and GMK. Echoviruses grew in HEF and RD-18S, and some in Vero and GMK. Growth of CoxA16 and entero71 was good in HEF and GMK, while it was poorer in Vero and RD-18S. Polioviruses replicated equally in all five cell lines.

DISCUSSION

First, the modified microplate method used in this study (HHVMRG plate) has all the merits of the original microplate method.[15] The original microplate method was developed and established in the mid-1980s. It has the following advantages: (1) isolation of a wide range of known respiratory viruses; (2) a fair isolation rate or a higher isolation rate than with the traditional tube method; (3) a simple and convenient procedure; and (4) a cost saving. We can use this method throughout the year for a large number of specimens, and this allows us to make a virologic diagnosis and to clarify the epidemiology of ARIs in children based on virus isolation.[17,18] However, a disadvantage of the modified microplate method is that the use of two extra cell lines decreases its simplicity and economy.

In the original HHVM plate, CoxA viruses, except for CoxA16, were never isolated.[15] This is because CoxA viruses are isolated well in newborn mice, and only some of them are cultivable in RD cell lines, especially in RD-18S.[5,10] In the HHVMRG plate, we succeeded in isolating CoxA10 in 2000 (data not shown), and CoxA2 and CoxA4 in 2001. Therefore, the isolation of the CoxA group, except for CoxA16, in RD-18S is one advantage of the new system. According to Sakae et al,[10] RD-18S is considered to be sensitive to CoxA2–6, 8, 10, 12, 17, 18, 21 and 24. RD cells are also assumed to be more sensitive than MK cultures to enteroviruses, except for CoxB, and are anticipated to represent a substitute for primary MK, which is not freely available.[9–11]

The sensitivity patterns of five cell lines (HHVRG) to enteroviruses have been deduced and are summarized in Table 3, based on the data shown in Table 2. As mentioned previously,[15] three groups of enteroviruses, CoxB, echo and polio, are distinguished from each other by a combination of three cell lines of HEF, HEp-2, and Vero. However, when isolates show CPEs in HEF and partly in Vero, it is difficult to differentiate CoxA16/entero71 from the echo group in that system. With a sensitivity test involving five cell lines, the difference in growth between RD-18S and GMK can easily distinguish these two groups. The CoxA group, except for CoxA16, is only sensitive to RD-18S, as described above. Finally, based on the sensitivity pattern of the HHVRG plate, we are able to roughly divide enterovirus isolates into five groups: (1) CoxA, except for CoxA16; (2) CoxA16/entero71; (3) CoxB; (4) echovirus; and (5) poliovirus. Such a presumptive identification allows us to go straight to the identical neutralization test with presumptive identification and

Table 2. Cell sensitivity of enteroviruses to five cell lines after a passage

Serotype	*Year*	*Specimen no.*	*HEF*	*HEp-2*	*Vero*	*RD-18S*	*GMK*
CoxA2	2001		×	×	×	○	×
CoxA4	2001		×	×	×	○	×
CoxA10	2000	845/1057	×	×	×	○	×
CoxA16	1997	451/494/496	○	×	□	×	○
CoxA16	1998	721/737/808	○	×	□	×	○
CoxA16	2000	933/939/1203	○	×	×□□	××□	○
CoxA16	2001		○	×	□	□	○
CoxB2	2001		×	○	○	×	○
CoxB3	2000	1207/1391/1408	×	○	○	×	○
CoxB3	2001		×	○	○	×	○
CoxB4	1999	998	×	○	○	×	○
CoxB5	1999	1099/1106	×	○	○	×	○
CoxB5	2001		□	○	○	×	○
Echo3	2000	1258/1259	○	×	□×	○	×
Echo6	1999	970/1001	○	×	□	○	○□
Echo9	1997	420/474/480	○	×	×□□	○	○
Echo11	1993	448/502/508	○	×	□	○	□□○
Echo16	2001		○	×	×	○	×
Echo17	2000	858/894/974	○	×	×	○	×□×
Echo18	1998	620/628/635	○	×	×	○	×
Echo25	2000	937/948/1061	□□○	×	×	○	××□
Echo30	1998	763/773/803	○	×	×	○	×
Echo30	1999	862/863	○	×	×	○	×
Entero71	1998	671/705	□○	×	□	□	○
Entero71	1999	932/957/983	○□○	×	□	□	○
Entero71	2000	934/962/1084	□○○	×	□	×□□	○
Entero71	2001		○	×	□	□	○
Polio1	2000	1164	○	○	○	○	○
Polio2	2000	1066	○	○	○	○	○
Polio2	2001		○	○	○	○	○

○, good growth; □, poorer growth than ○; ×, no growth.
When sensitivity to the cell lines was different among the isolates, all patterns are shown in order.

Table 3. Cell sensitivity pattern of enterovirus groups after a passage

Groups of enteroviruses	*HEF*	*HEp-2*	*Vero*	*RD-18S*	*GMK*
CoxA except for CoxA16	×	×	×	○	×
CoxA16/Entero71	○	×	□	□	○
CoxB	□	○	○	×	○
Echo	○	×	□	○	□
Polio	○	○	○	○	○

○, good growth; □, poorer growth than ○; ×, no growth.

to avoid using irrelevant antisera. For example, when an isolate is presumptively identified as belonging to the CoxB group, the isolate should be neutralized with antisera against CoxB1–6. Accordingly, the presumptive identification saves money, time and labor, compared with the classical neutralization test.

The cell sensitivity pattern of primary inoculation does not always coincide with the one shown in Table 3. For example, in a primary inoculation, four isolates of echo16 only showed CPEs in RD-18S, and one in HEF, as shown in Table 1. Therefore, to work as a presumptive identification method, the cell sensitivity test with HHVRG plate should preferably be carried out after a passage. The cell sensitivity pattern after a passage for CoxA except for CoxA16 and poliovirus is perfectly stable. However, we have to admit that there is a variability in cell sensitivity pattern among the CoxA16/entero71, CoxB and echovirus groups. This is because of the intrinsic variability of wild enterovirus strains, and the existence of such diversity places limits on presumptive identification.

In the last decade, RT-PCR-based methods have been more widely used in enteroviral diagnosis.[16,19–22] Particularly when our aim is limited to detecting enteroviruses, these methods have advantages of rapidity and sensitivity compared with virus isolation.[16,19,20] This represents a considerable benefit for clinical diagnostic use. Currently, even serotyping by sequence analysis is available.[20–22] We do not intend to deny the benefits of modern molecular tools. However, in reality, causative viral agents of ARI are not only enteroviruses but also influenza viruses, adenoviruses, RS viruses, parainfluenza viruses, etc.[15] We have detected dual viral infections in the same patient.[18] The microplate method allows us to isolate enteroviruses not only in the hot summer season, which is the peak time for enteroviral infections in the temperate zone, but also in the winter season.[15] To detect such a wide range of respiratory viruses using PCR for DNA viruses and RT-PCR for RNA viruses throughout the year is expensive, laborious, and complicated. Therefore, we would like to emphasize that the microplate method for the clarification of the epidemiology of ARI still has validity. Of course, we should introduce molecular tools, especially to perform further analyses for untyped enteroviruses.[22]

The combined use of several cell lines has been employed to isolate enteroviruses efficiently.[6–9,11–14] There is no perfect combination that covers all serotypes of enteroviruses. The modified microplate method (HHVMGR plate) proposed here is one of the alternatives. The HHVMRG plate method can isolate some of the CoxA group viruses, CoxB, echoviruses, entero71, polioviruses and even other respiratory viruses. Therefore, this method may be appropriate for the surveillance of ARI. We expect that this method will be used routinely and will clarify the epidemiology of ARI in children, especially that of enterovirus infections.

ACKNOWLEDGEMENTS

We thank the doctors, nurses and people in Yamagata Prefecture for assisting and collaborating in the surveillance of viral infectious diseases. This work was partially supported by DAIDO life welfare foundation.

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