Journal of Medical Microbiology (2008), **57**, 1000–1006 DOI 10.1099/jmm.0.47799-0

A convenient rapid culture assay for the detection of enteroviruses in clinical samples: comparison with conventional cell culture and RT-PCR

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Received 6 December 2007
Accepted 24 March 2008

A convenient rapid culture assay (RCA) for the detection of enteroviruses was evaluated against RT-PCR using 576 stool and 102 cerebrospinal fluid (CSF) samples. One hundred and ninety stool samples were also tested by conventional cell culture (CCC). The RCA used immunoperoxidase staining of cell culture plates with a blend of monoclonal antibodies (mAbs) against enterovirus VP1 on the second and sixth days after inoculation. This blend was composed of 5D8/1 (Dako) and four 'in-house' mAbs. CCC was performed using fluorescence staining with the Enterovirus Screening Set (Chemicon International) for culture confirmation. Detection of enteroviruses by the RCA was more successful in colonic carcinoma (CaCo-2) and rhabdomyosarcoma (RD) cells than in human embryonic lung fibroblasts, HEp2 and A549 cells. The performance of CCC in RD cells was hindered by rapid cell degeneration and non-specific staining of cells during culture confirmation. The sensitivity of the RCA compared to RT-PCR in stool samples was found to be 71 % (115/161) on the second day and 87 % (140/161) on the sixth day. The sensitivity of the RCA in CSF samples was 38 % (22/58) after 2 days and 59 % (34/58) after 6 days. The specificity of the RCA was 100 %. All CCC-positive samples were positive by the RCA. CCC required 3–14 days for virus recovery. In conclusion, the RCA has the same sensitivity as CCC, significantly shortens the time required for the detection of enteroviruses, and prevents pitfalls associated with using RD cells for CCC. For diagnosis of aseptic meningitis in CSF samples, RT-PCR should be performed.

INTRODUCTION

RT-PCR is the most sensitive and rapid method for detection of enteroviruses. However, cell culture is still used in clinical laboratories for diagnosis for reasons associated with cost and availability, as well as for its ability to identify different serotypes for surveillance and environmental purposes. According to current virus classification, human enteroviruses are divided into five species within the genus *Enterovirus*: *Poliovirus* (PV-1, -2 and -3), *Human enterovirus A* (HEV-A) (coxsackievirus A2, 3, 5, 7, 8, 10, 12, 14 and 16 and enterovirus 71), HEV-B (coxsackievirus A9, coxsackievirus B 1–6, echovirus 1–7, 9, 11–21, 24–27, 29–33 and enterovirus 69), HEV-C (coxsackievirus A1, 11, 13, 15, 17–22 and 24) and HEV-D (enterovirus 68 and 70) (King *et al.*, 2000). Single cell lines cannot support the growth of all of these different enterovirus types; therefore, a combination of cell lines must be used. However, even though cell culture became a routine laboratory technique more than 50 years ago, there is still no consensus concerning the optimal protocol for isolation of enteroviruses (Table 1). Due to the fact that virus isolation in conventional cell culture (CCC) normally takes 3–14 days, rapid shell vial (SV) culture techniques utilizing commercially available monoclonal antibodies (mAbs) for fluorescence staining have been developed (Klespies *et al.*, 1996; Van Doornum & De Jong, 1998; Lipson *et al.*, 2001; Perez-Ruiz *et al.*, 2003). Although SV assays reduce the turnaround time for diagnosis, there are major disadvantages, including tedious manual preparation and washing of cover slips. A more convenient rapid cell culture technique using microwell cell cultures and immunoperoxidase staining with 5-D8/1 mAb has been described by Bourlet *et al.* (1998). According to literature published up until now, this staining technique for detection of enteroviruses is not widely used (Table 1).

In all of the aforementioned studies, the sensitivity of SV assays and rapid cell culture techniques was compared with that of CCC, but not with that of RT-PCR.

Abbreviations: CCC, conventional cell culture; CPE, cytopathic effect; CSF, cerebrospinal fluid; RCA, rapid culture assay; RKI, Robert Koch Institute; WHO, World Health Organization.

Table 1. Methods for detection of enteroviruses used in different laboratories

Abbreviations: HEL, human embryonic lung fibroblasts; KB, human epidermoid carcinoma cells; MRC-5, human diploid fibroblasts; PMK, primary rhesus monkey kidney; t-MK, tertiary cynomolgus monkey kidney; BGM, buffalo green monkey kidney; RD, embryonic rhabdomyosarcoma; CaCo-2, colonic carcinoma; RMK, rhesus monkey kidney; SF, human fetal foreskin cells; DAF-BGM, genetically engineered BGM expressing human decay-accelerating factor; CCC, conventional cell culture in tubes; SV, shell vial; MCC, microplate centrifugation culture; CPE, cytopathic effect; NT, neutralization test; IFA, immunofluorescence assay; PCE, pre-cytopathic effect; IPS, immunoperoxidase staining.

Reference	Cell lines	Cultural methods and enterovirus identification	mAb used for IFA or IPS
Trabelsi *et al.* (1995)	HEL, KB	CCC (CPE, NT, IFA, PCR)	5-D8/1 (Dako)
Klespies *et al.* (1996)	MRC-5, PMK	CCC (CPE, IFA); SV (PCE, IFA)	Enterovirus mAbs (Chemicon International)
Bourlet *et al.* (1998)	HEL, KB	CCC (CPE, IFA, NT); MCC (PCE, IPS)	5-D8/1 (Dako)
Kok *et al.* (1998)	BGM, A549, HEL, RD	CCC in microplates (CPE, NT)	
Van Doornum & De Jong (1998)	t-MK, HEL	CCC (CPE, NT); SV (PCE, IFA)	5-D8/1 (Dako)
Lipson *et al.* (2001)	A549, RMK, MRC-5, HEp2,	CCC (CPE, IFA); SV (PCE, IFA)	Pan-Enterovirus 2E11 (Chemicon International) 5-D8/1 (Dako)
Otero *et al.* (2001)	A549, MRC-5, BGM	CCC (CPE, NT)	
Buck *et al.* (2002)	SF, RMK, A549; SV: DAF-BGM mixed with CaCo-2	CCC (CPE, IFA); SV (PCE, CPE, IFA)	Pan-Enterovirus Blend (Chemicon International)
Perez-Ruiz *et al.* (2003)	RD, MRC-5, Vero	CCC (CPE, IFA); SV (PCE, IFA)	5-D8/1 (Dako)
She *et al.* (2006)	PMK, BGM, RD, A549, MRC-5	SV (CPE, IFA, NT)	Enterovirus mAbs (Chemicon International)

In the present study, the applicability of different cell lines and different mAbs for a rapid culture assay (RCA) were investigated. Also, we investigated the diagnostic performance of the newly developed RCA and compared it with that of CCC and RT-PCR.

METHODS

Reference viruses. The following enterovirus strains were used for evaluation of mAb reactivity: coxsackievirus type A5 (CV-A5, G.S., Swartz VR164), coxsackievirus type A7 (CV-A7, AB-IV, Russian VR319), coxsackievirus type A9 (CV-A9, P.B., Bozek, VR186), coxsackievirus type A10 (CV-A10, M.K., Kowalik, VR168), coxsackievirus type A16 (CV-A16, G-10, VR174), coxsackievirus type B1 (CV-B1, Conn-5, VR28), coxsackievirus type B2 (CV-B2, Ohio, VR29), coxsackievirus type B3 (CV-B3, Nancy, VR30), coxsackievirus type B4 (CV-B4, J.V.B., Bebschoten, VR184), coxsackievirus type B5 (CV-B5, Faulkner, VR185), coxsackievirus type B6 (CV-B6, Schmitt, VR155), echovirus 7 (E-7, Wallace,VR1047), echovirus 9 (E-9, Hill, VR1050), echovirus 14 (E-14, Tow, VR1055), echovirus 20 (E-20, JV-1, VR1061), echovirus 30 (E-30, Bastianni, VR1072), enterovirus 71 (EV-71, BrCr, VR784) obtained from the ATCC, Sabin attenuated strains of poliovirus types 1, 2 and 3 (PV-1, PV-2, PV-3) obtained from Behringwerke, and 'in-house' isolated strains of coxsackievirus type A2 (CV-A2), echovirus 6 (E-6), echovirus 11 (E-11) and echovirus 13 (E-13), which were typed at the Robert Koch Institute (RKI, national reference centre) by neutralization tests using RKI and World Health Organization (WHO) pools of specific antisera.

Clinical specimens. Stool and cerebrospinal specimens were obtained from general practitioners and from paediatric hospitals. They originated from patients suspected of having an enterovirus infection based on symptoms including meningitis, encephalitis, hand, foot and mouth disease and diarrhoea.

Processing of samples. A small amount of stool (0.5–1 g) was resuspended in 5 ml PBS, centrifuged (10 min, 4000 ***g***), and filtered through 0.45 μm filters. Cerebrospinal fluid (CSF) specimens were inoculated directly into cells.

Cell lines. The following cell lines were used: human embryonic lung fibroblasts (HEL) ('in-house'), human colonic carcinoma cells (CaCo-2), embryonic rhabdomyosarcoma (RD) cells, HEp-2 cells and human Caucasian lung carcinoma cells (A-549) (European Collection of Cell Cultures).

mAbs. The following were used: Enterovirus Screening Set, including Echovirus Blend, Enterovirus Blend, Coxsackie Blend, Poliovirus Blend and Pan-Enterovirus Blend (Chemicon International); Coxsackievirus Antibody Set, including antibodies against coxsackievirus B1–6, A9 and A24 (Chemicon International); Echovirus Antibody Set, including antibodies against echovirus 4, 6, 9, 11 and 30 (Chemicon International); 5-D8/1 (Dako); clones SEV1, SEV2, SEV3 and SEV4 (in-house). The reactivity of some antibodies is shown in Table 2.

CCC. Cell culture tubes with CaCo-2, RD and HEL cells were inoculated with samples according to standard protocols (Wiedbrauk & Johnston, 1993). Tubes were checked up to 10 days for a cytopathic effect (CPE). If RT-PCR-positive stool samples were negative on day eight, blind passages were done and cultures were examined for at least 14 days. Cytopathic changes within 24 h were regarded as toxic. Confirmation of enterovirus infection in cell cultures with CPE was performed by immunofluorescence staining using an Enterovirus Screening Set, according to the manufacturer's instructions.

Typing of viruses. Typing of viruses was performed in cells with clearly visible enterovirus CPE using the immunofluorescence method as described by Rigonan *et al.* (1998). Stool samples where the enteroviruses could not be typed by immunofluorescence were typed

Table 2. Reactivity of different mAbs with reference enteroviruses

Detection of reactivity of 5-D8/1 (Dako), SEV1, SEV2, SEV3 and SEV4 ('in-house') was carried out by immunofluorescence and immunoperoxidase staining for all virus types. Detection of reactivity of the Pan-Enterovirus Blend (Chemicon International) was carried out by immunofluorescence for all virus types and by immunoperoxidase staining for CV-A16, CV-B5 and E-9. Dilutions of mAbs used: Pan-Enterovirus Blend, undiluted; 5-D8/1, 1 : 100; SEV1, SEV2, SEV3 and SEV4, 1 : 10. +, Positive; −, negative.

Enterovirus serotype	Pan-Enterovirus Blend	5-D8/1	SEV1	SEV2	SEV3	SEV4
EV-71	+	−	+	+	+	−
CV-A2	+	−	+	+	−	−
CV-A5	+	−	−	+	+	−
CV-A7	+	−	+	+	−	−
CV-A9	+	+	−	−	−	−
CV-A10	+	−	−	+	+	−
CV-A16	+	−	+	+	+	−
CV-B1	+	+	+	−	−	−
CV-B2	+	+	−	−	−	+
CV-B3	+	+	+	−	−	+
CV-B4	+	+	+	−	−	+
CV-B5	+	+	+	−	−	−
CV-B6	+	+/−	−	−	−	−
E-2	+	+	+	+	−	−
E-6	+	+	+	+	−	−
E-7	+	+	+	+	−	−
E-9	+	+	+	+	−	−
E-11	+	+	+	+	−	−
E-13	+	+	−		−	−
E-14	+	+	+	+	−	−
E-20	+	+	+	+	−	−
E-30	+	+	+	+	−	−
PV-1	+	+	−	−	−	−
PV-2	+	+	−	−	−	−
PV-3	+	+	−	−	−	−

by neutralization tests using Lim Benyesh-Melnik pools of type-specific antisera (Statens Serum Institut, Copenhagen, Denmark) or sent to the RKI (national reference centre) and typed by neutralization tests using RKI and WHO pools of specific antisera.

RCA. Different cell lines were seeded separately into 24-well microtitre plates in Eagle's modified essential medium with 2 % FCS 1–3 days before testing (1 ml per 100 000 cells per well). Wells were inoculated with 100 µl sample, centrifuged at 800 ***g*** for 45 min, and incubated in a moist chamber at 37 °C with 5 % CO_2 in air. The plates were stained on the second and sixth day post-infection. For staining, cells were fixed for 10 min with ice-cold acetone/methanol (40 : 60), blocked with 1 % (w/v) BSA in PBS for 30 min, and stained using the immunoperoxidase method. The mixture of mAbs for staining contained 5-D8/1, SEV1, SEV2, SEV3 and SEV4. The plates were incubated for 30 min at 37 °C with mAb (0.2 ml per well), then with secondary horseradish peroxidase-labelled anti-mouse antibody (anti-mouse-HRP; Dako), and finally with the substrate 3-amino-9-ethylcarbazole (Sigma). Stained cells were examined with a light microscope. Prior to staining, cell culture supernatants were collected and, if positive, passaged into cell culture tubes with fresh cells for further typing.

Comparison of cell line susceptibility. Tenfold dilutions of viruses were made in order to determine the end-point detection limit for the growth of different enterovirus types in different cells. CaCo-2, RD, HEp-2 and A549 cells were each seeded into two 96-well microtitre plates. Plates were then inoculated with the virus dilutions. One microtitre plate was stained the next day post-infection using a mixture of antibodies (5-D8/1, SEV1, SEV2, SEV3 and SEV4). The other plate was used for examination of CPE 6 days post-infection.

Real-time RT-PCR. Real-time RT-PCR was performed using the following primers: Ent F (5′-GGC CCC TGA ATG CG-3′) and Ent R (5′-CGG ATG GCC AAT CCA-3′) derived from the 5′NCR (Chapman *et al.*, 1990). The detection probe was a 25-mer oligonucleotide, Ent TM (FAM-5′-ACA CGG ACA CCC AAA GTA STC GGT T-3′-TAMRA) (TIB MolBiol). A typical 20 µl reaction mixture contained 10 µl QuantiTect Probe Master mix (Qiagen), 0.5 µM of each primer, 0.18 µM probe, 0.2 µl QuantiTect RT mix and 6.8 µl of the purified RNA. Real-time RT-PCR was carried out with an initial RT step at 50 °C for 20 min, followed by PCR activation at 95 °C for 15 min, 10 cycles of amplification at 95 °C for 5 s, 65 °C for 20 s and 72 °C for 15 s, and 40 cycles of amplification at 95 °C for 0 s, 55 °C for 20 s and 72 °C for 30 s. Fluorescence development was measured once every cycle after the elongation step.

RESULTS AND DISCUSSION

Choice of mAbs for virus identification

Currently, there are two commercially available reagents against common enterovirus epitopes: Pan-Enterovirus

Blend (mixture of 9D5 and 2E11 mAbs), presumably directed against a virus-encoded, non-virion determinant (Yagi *et al.*, 1992), and 5-D8/1, which reacts with the VP1 polypeptide. 'In-house' mAbs SEV1, SEV2, SEV3 and SEV4 are also directed against VP1 epitopes, as was proved by Western blotting (data not shown). The reactivity of these mAbs to different reference viruses tested by immunofluorescence and immunoperoxidase staining is shown in Table 2. The Pan-Enterovirus Blend reacted with all enterovirus types and was therefore the ideal candidate for the RCA, but could only be used undiluted, which made the RCA assay in 24-well plates very expensive. The 5-D8/1 mAb could be used at a dilution of 1 : 100, but failed to react with coxsackievirus A2, A4, A5, A6, A10 and A16 and enterovirus 71, which belong to the HEV-A species. The only coxsackievirus A which was identified by 5-D8/1 mAb was coxsackievirus A9. This enterovirus belongs to the species HEV-B. Additional use of other antibodies in the staining mixture for the RCA, such as SEV1, SEV2 and SEV3, which recognize species HEV-A, was necessary. Thus a mixture of 5-D8/1, SEV1, SEV2, SEV3 and SEV4 was used for the RCA. Nevertheless, the CCC was performed using the Pan-Enterovirus Blend, because this reagent has the brightest reactivity spectrum and has been well established for culture confirmation (Table 1).

Choice of cell lines

At the present time, there is no general agreement concerning the optimal panel of cell lines for isolation of enteroviruses (Table 1). Thus the applicability of different cell lines for the RCA was investigated.

Overall, CaCo-2 and RD cells yielded higher virus titres than HEp-2, A549 and HEL cells. The RD and CaCo-2 cells supported the growth of coxsackievirus A7, A10 and A16 and echovirus 6, 7 and 20 at the same titre (Table 3). Echovirus 9, 11 and especially 14 grew better in RD cells. Coxsackie B viruses grew better in CaCo-2 cells. Coxsackie B viruses could also be detected by immunoperoxidase staining in plates with RD cells the next day, but the CPE was not observed even 6 days after infection.

Detection of enterovirus in clinical specimens by the RCA in 2005 was carried out using CaCo-2, RD and HEL cells. During the 2006 and 2007 seasons, HEp2 cells were used as a third cell line in addition to CaCo-2 and RD cells for performing the RCA. CaCo-2 recovered the majority of enteroviruses (Table 4). HEL and HEp2 did not improve the total result. Only coxsackie B viruses grew successfully in HEp2 cells. All samples containing coxsackie B viruses were also positive in CaCo-2 cells. CaCo-2 cells yielded more echovirus 11 and 30 and enterovirus 71 isolates than RD cells. RD cells were more effective in the isolation of coxsackie A viruses 6, 10 and 16 and some echoviruses.

Table 3. Virus titre (log_{10} of the reciprocal end-point dilution) of different entrovirus types found in RD and CaCo-2 cells by immunoperoxidase staining (IPS) and by observing CPE on the first and sixth days post-infection

Enterovirus type	RD (IPS) 1st day	RD (IPS) 6th day	RD (CPE) 6th day	CaCo-2 (IPS) 1st day	CaCo-2 (CPE) 6th day
CV-A7	7		7	7	7
CV-A9	6		6	5	5
CV-A10	6		6	6	6
CV-A16	5		5	5	5
CV-B1	6	6	0	6	6
CV-B2	3	6	0	6	6
CV-B3	4	5	0	6	6
CV-B4	5	5	0	6	6
CV-B5	2	4	0	4	4
CV-B6	6	6	0	6	6
E-6	7		7	7	7
E-7	7		7	7	7
E-9	7		7	5	5
E-11	6		6	5	5
E-14	5		5	2	2
E-20	5		5	5	5
E-30	4		4	3	3

Table 4. Enterovirus types detected by the RCA in clinical samples using CaCo-2 and RD cell lines

Enterovirus type	Cell line		No. of isolates (total)
	CaCo-2	RD	
CV-A2	2	2	2
CV-A4	3	3	3
CV-A5	2	2	2
CV-A6	0	1	1
CV-A9	6	7	7
CV-A10	0	1	1
CV-A16	0	2	2
CV-B1	2	1	2
CV-B2	11	7	12
CV-B3	7	6	7
CV-B4	2	1	2
CV-B5	16	9	17
E-2	2	3	3
E-4	11	18	18
E-5	0	1	1
E-6	8	8	8
E-9	5	6	6
E-11	23	19	23
E-13	1	1	1
E-14	2	2	2
E-18	7	9	9
E-25	5	5	5
E-30	42	40	42
EV-71	14	10	16
Untyped enteroviruses	3	2	4
Total	174	166	196

Comparison of the sensitivity and specificity of the RCA and CCC

The RCA was evaluated in comparison to CCC using a total of 190 stool samples, which were also tested by RT-PCR.

Prior to routine laboratory testing of the RCA, a small set of 31 RT-PCR-positive samples was used for comparison of the RCA and CCC. These samples were frozen, kept at −70 °C for 6 months, and after thawing were tested by CCC and the RCA (both CaCo-2, RD and HEL cells). Twenty-two samples were positive by both CCC and the RCA.

During enterovirus season 2005, a total of 159 stool samples were routinely tested by CCC, the RCA (both, CaCo-2, RD and HEL cells) and RT-PCR. Twenty-nine stool samples were positive by RT-PCR. Twenty-five of those samples were positive by both CCC and the RCA. Thus the sensitivity of the RCA in comparison to that of CCC in both sample sets was 100 % (47/47).

CCC results were positive for seven out of 130 RT-PCR-negative samples (one was a CaCo-2 isolate and six were RD isolates). CPE in cell cultures inoculated with all of these samples was confirmed by immunofluorescence staining with the Pan-Enterovirus Blend. The CaCo-2 isolate was positive by astrovirus EIA. Six isolates from RD cells reacted positively with the Pan-Enterovirus Blend and also slightly with the Echovirus 30 Reagent from the Echovirus Antibody Set. All samples were negative by the RCA. Attempts to passage virus from these samples failed. Thus these seven samples were false-positives by CCC. The reduced specificity of the CCC could be explained by the cross-reactivity of the mAb used. mAb 9D5, the first component of the Pan-Enterovirus Blend, reacts with all enterovirus types, but according to the data sheet also cross-reacts with hepatitis A, astrovirus, reovirus 3, rhinovirus 30, adenovirus 31 and adenovirus 48, and slightly with cultured cells. The same applies to mAb 2E11, the second component of the Pan-Enterovirus Blend. Non-specific enterovirus staining was observed particularly in RD cells. The WHO recommends RD cells for detection of polio and non-polio enteroviruses (WHO, 2004). However, use of this cell line can hide some pitfalls. Clear CPE patterns are necessary to identify preliminarily the virus in CCC as an enterovirus. Kok *et al.* (1998) observed that RD cells show non-specific cytopathic changes earlier than other cell lines. Our experience confirmed this. Immunofluorescence staining of cells with the Pan-Enterovirus Blend was expected to distinguish specific from non-specific cytopathic changes. However, the cross-reaction of antibodies to quickly degenerating RD cells was mistaken for positive staining. Faden *et al.* (2006) also had a similar problem with the quickly degenerating cell line A549. The WHO recommends establishing conditions (adjustment of cell count and concentration of fetal calf serum) that will allow cells to last for at least 5–7 days without degeneration (WHO, 2004). However, many isolates require incubation times longer than 7 days. The WHO makes appropriate recommendations for blind passages for a minimum of 14 days by following culture confirmation with typing sera. These recommendations were generated for surveillance purposes, but clinical practice requires more rapid diagnosis.

In this study, only 6 % of samples were found to be positive by CCC on day 3. The proportion of specimens positive by CCC after 6 days of incubation was 68 %. The remaining 32 % of samples required 7–14 days of incubation for positive virus detection. It also must be mentioned that these results were achieved mainly in CaCo-2 cells. Isolation of enteroviruses by CCC in RD cells normally required additional passages and took more than 7 days. By comparison, the RCA detected 87 % (41/47) of CCC-positive stool samples on day two and 100 % on day six. Thus the RCA significantly shortened the time required for the detection of enteroviruses. Moreover, the RCA prevented the pitfalls associated with using RD cell culture followed by confirmation with the Pan-Enterovirus Blend.

Comparison of the sensitivity and specificity of RCA and RT-PCR

In conjunction with the non-polio enterovirus surveillance project started in October 2005 by the National Commission for Polio Eradication in Germany (Robert Koch-Institut, 2006), all enterovirus RT-PCR-positive samples were subsequently cultured and the isolated virus was typed. In 2006–2007, isolation of enterovirus was only done by the RCA (CaCo-2, RD and HEp2 cells) and positive supernatants were passaged in fresh cells in tubes for further typing. Overall, from 161 RT-PCR-positive stool samples, 115 (71 %) were positive after the second day of inoculation and 140 (87 %) were positive after the sixth day (Table 5).

The sensitivity of the RCA in CSF samples was lower than in stool samples. A total of 102 CSF samples were tested

Table 5. Sensitivity of the RCA compared to that of RT-PCR (5′ non-coding region)

Immunostaining of RCA plates was carried out on the second and sixth day post-infection.

Year	Sample	No. positive by RT-PCR	No. (%) positive by RCA	
			2nd day	6th day
2005	Stool	29	21 (72)	25 (86)
	CSF	7	5 (71)	5 (71)
2006	Stool	63	47 (75)	56 (88)
	CSF	41	14 (34)	24 (59)
2007	Stool	69	47 (68)	59 (86)
	CSF	10	3 (30)	5 (50)
Total	Stool	161	115 (71)	140 (87)
	CSF	58	22 (38)	34 (59)

(2005–2007). Fifty-eight samples were found to be positive by RT-PCR. The sensitivity of the RCA was 38 % (22/58) after 2 days and 59 % (34/58) after 6 days.

The specificity of the RCA versus RT-PCR was 100 % in stool (384/384) and CSF (44/44) samples.

The majority of studies that have evaluated the diagnostic performance of rapid virus detection in cell culture used CCC, but not PCR, as a reference method. The overall sensitivity in these studies can be overestimated because some enteroviral viruses do not grow in cell culture. Only one study (Buck *et al.*, 2002) that included CSF specimens compared the sensitivity of SV assay with that of RT-PCR and found it to be 76 %. In our study, the sensitivity of the RCA versus PCR was lower in CSF specimens but higher in stool specimens. However, when comparing the sensitivities of the different studies, the types of circulating enterovirus strains with a strong influence on the sensitivity of cell culture should be considered. In years with high activity of types growing well in cell culture, such as in 2000, the sensitivity of cell culture can be higher than in years with lower enterovirus activity (Roth *et al.*, 2007).

Enterovirus types identified by the RCA in clinical samples

A total of 24 different enteroviruses were found in 196 RCA-positive clinical samples. Enterovirus serotypes detected in stool and CSF samples by the RCA are shown in Table 4.

mAb 5-D8/1 did not react with HEV-A group enteroviruses from clinical samples as with reference strains. Lack of 5-D8/1 reactivity with enterovirus 71 and coxsackie A viruses was also described by Schnurr *et al.* (1996). Thus epidemiologically important enterovirus types such as coxsackievirus A16 and enterovirus 71 causing hand, foot, and mouth disease cannot be detected using mAb 5-D8/1. This deficit in reactivity must be considered in laboratories using this reagent. Additional use of other antibodies in the staining mixture, such as SEV1, SEV2 and SEV3, which recognize the HEV-A group is essential for an effective RCA.

In conclusion, our data show that the RCA is not only more rapid than CCC, but also helps to avoid the pitfalls associated with using RD cells for CCC. CaCo-2 and RD cells are more effective than HEL, HEp2 and A549 cells in detecting enteroviruses by the RCA. If RT-PCR is not available, the RCA can be used for detection of enteroviruses in clinical samples. In this case, stool specimens are preferred. For diagnosis of aseptic meningitis in CSF samples, RT-PCR should be performed.

ACKNOWLEDGEMENTS

The authors thank Dr S. Diedrich of the Robert Koch Institute, Berlin, for assistance with the serotyping of some enterovirus isolates.

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