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NOTES

Serotype-Specific Detection of Coxsackievirus A16 in Clinical Specimens by Reverse Transcription-Nested PCR

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We describe the development of a coxsackievirus A16 (CVA16) serotype-specific PCR which correctly differentiated between CVA16 and other enterovirus serotypes of both laboratory isolates and clinical specimens. The assay will be useful for monitoring CVA16 outbreaks and studying the disease association, epidemiology, and evolution of this common enterovirus serotype.

Enteroviruses comprise a large genus within the *Picornaviridae* which includes the polioviruses, group A and B coxsackieviruses, and echoviruses. Common human pathogens, they are traditionally diagnosed by virus isolation in cell cultures and, in the case of coxsackieviruses, by suckling mouse inoculation (SMI). Serotypic identification of enterovirus isolates is not usually required for patient management but is useful in studying enterovirus outbreaks; in addition, it is required to differentiate between wild or vaccine-derived polioviruses and nonpolioviruses in cases of acute flaccid paralysis (4, 5) or, more commonly, to characterize enterovirus isolates from cases of aseptic meningitis as a means of achieving certification of poliovirus eradication (14). Serotyping is achieved by neutralization of viral infectivity in cell cultures or suckling mice using individual or pooled serotype-specific neutralizing antisera or by indirect immunofluorescence using serotype-specific monoclonal antibodies. However, these methods lack sensitivity. SMI is most sensitive for coxsackievirus isolation, but it is not widely available and is slow, labor-intensive, and ethically undesirable.

In recent years, molecular diagnostic methods have been increasingly used for enterovirus diagnosis. Although sensitive, these methods do not generally allow serotype identification. There is therefore a need for additional molecular tools for serotype or genotype identification of enteroviruses which complement existing PCR methods for generic enterovirus detection (reviewed in reference 10). The development of such assays has been limited thus far (1, 3, 6, 9, 12, 16) and, as most have been used to study enterovirus isolates rather than clinical specimens, they cannot yet completely replace traditional isolation methods.

Coxsackievirus A16 (CVA16) is the most commonly detected of the group A coxsackieviruses (CVA) and is best known for causing hand, foot, and mouth disease (HFMD). Although normally benign, fatal central nervous system complications have been observed in recent epidemics of HFMD

TABLE 1. Primers used in the CVA16-specific PCR and generic enterovirus-reactive PCR

PCR	Primer	Use	Nucleotide position on CVA16 genome[a]	Region of genome	Sequence (5′→3′)
CVA16 specific	Outer upstream	First-round PCR	3127–3148	VP1	ACC ATG ATG GGC ACT TTT AGC A
	Outer downstream	Reverse transcription and first-round PCR	3306–3286		GGT GCA CTT GAT ATC ATT TCC
	Inner upstream	Nested PCR	3162–3182		GAC TGA AAA GTC ACC ACA ITC
	Inner downstream	Nested PCR	3270–3251		GGT CTT GAA CAA ATA GGG TTG
Generic enterovirus	Outer upstream	First-round PCR	457–473	5′ Untranslated region	CGG CCC CTG AAT GCG GC
	Outer downstream	Reverse transcription and first-round PCR	649–632		CAC CGG ATG GCC AAT CC A
	Inner upstream	Nested PCR	460–477		CCC CTG AAT GCG GCT AAT
	Inner downstream	Nested PCR	606–587		ATT GTC ACC ATA AGC AGC CA

[a] CVA16 G10 prototype genome.

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TABLE 2. Enterovirus isolates tested with the CVA16-specific PCR

Isolate(s)[a]	No. positive/ no. tested
CVA16 G10 prototype	1/1
CVA16 laboratory isolates, 1980–2000	21/21
Other CVA prototypes: serotypes 1–15, 17–22, and 24	0/22[b]
CVA9 laboratory isolates, 1990–1999	0/8
CVB serotypes 1–5 (BioWhittaker Ltd.)	0/5
Poliovirus serotypes 1–3 vaccine strains	0/6
Echovirus serotypes 1, 2, 4–9, 11–21, 23, 24, 26, 27, 29, and 30; laboratory isolates, and CPHL strains	0/24
EV71 laboratory isolates, 1998–2000	0/4
Rhinovirus laboratory isolate, 1999	0/1

[a] CPHL, Central Public Health Laboratory, London, United Kingdom.
[b] CVA14 produced a positive result on first-round PCR.

caused by enterovirus 71 (EV71), a serotype closely related to CVA16, in the Far East (7, 15). Identifying the cause of HFMD may thus be of prognostic and epidemiologic value. Because CVA16 is common, rapid molecular identification would also reduce the number of enterovirus isolates requiring serological typing. We have therefore developed and evaluated a CVA16-specific nested PCR.

RNA was extracted from specimens using RNAzol B (Biogenesis Inc., Poole, Dorset, United Kingdom). The presence of enteroviral RNA was determined using generic enterovirus-reactive primers, and CVA16 RNA was detected using CVA16-specific primers (Table 1). Published viral capsid protein (VP1)-coding sequences of CVA16 (13) and the closely related EV71 (2), as well as VP1 sequence data for five additional English CVA16 isolates and one English EV71 isolate collected during the last 15 years, were used to design CVA16-specific primers, since VP1 sequences show the greatest correlation with serotype (3). Reverse transcription, first-round PCR, and nested PCR reagents and amplification conditions were the same for both assays and were essentially as described elsewhere (11). First- and second-round PCR products were visualized by agarose gel electrophoresis.

Twenty-two CVA16 isolates, including 20 English isolates collected between 1980 and 2001, were used to evaluate the CVA16-specific PCR. In addition, at least one example of each of the 23 CVA serotypes (mostly prototype strains) and isolates of other enterovirus serotypes were tested. Isolates were cell culture supernatants or suckling mouse torso suspensions and had been stored at −70°C or −20°C prior to testing. All of the CVA16 isolates gave positive results with both the outer set of primers and the inner (nested) set of primers; none of the non-CVA16 isolates gave positive results following nested PCR (Table 2), although a CVA14 isolate gave a positive result following first-round PCR. This result presumably reflects sequence similarity between CVA16 and CVA14 within these outer primer recognition sequences and emphasizes that only when both primer sets are used in a nested PCR can the assay be considered serotype specific. The assay was also evaluated using 22 clinical specimens, including 16 which yielded CVA16 by cell culture or SMI and 6 which yielded other enteroviruses. All 16 specimens containing CVA16 tested positive following CVA16-specific nested PCR, although one was only weakly positive; no specimens found to contain other enterovirus serotypes tested positive (Table 3).

TABLE 3. Clinical specimens tested with the CVA16-specific PCR

Clinical specimen(s)	Enterovirus isolated by cell culturing or SMI	No. positive/ no. tested	Clinical illness
Stools	CVA16	13/13	HFMD
Lip swab	CVA16	1/1	HFMD
Mouth swabs	CVA16	2/2	HFMD
Stool	CVB3	0/1	Febrile neutropenia
Stool	CVA2	0/1	Myocarditis (fatal)
Stool	EV71	0/1	HFMD
Stool	Poliovirus serotype 2	0/1	None (vaccinee)
Stool	Echovirus serotype 13	0/1	Aseptic meningitis
Stool	Echovirus serotype 7	0/1	Cot death

TABLE 4. Sensitivity of the CVA16-specific PCR

Virus dilution[a]	No. of infected samples/ no. tested	PCR result[b]
None (CVA16 stock CCS)	—	—[c]
10^{-1}	4/4	+
10^{-2}	4/4	+
10^{-3}	4/4	+
10^{-4}	4/4	+
10^{-5}	0/4[d]	+
10^{-6}	0/4	+
10^{-7}	0/4	−

[a] CCS, cell culture supernatant.
[b] +, positive; −, negative. The limit of detection of the PCR was the 10^{-6} dilution or $10^{-1.5}$ (0.03) $TCID_{50}$ per 100 μl.
[c] —, not tested.
[d] $TCID_{50}$ calculated as $10^{-4.5}$ per 100 μl.

The sensitivity of the CVA16-specific PCR relative to cell culture isolation was determined by testing serial 10-fold dilutions of a recent CVA16 isolate in parallel by CVA16-specific PCR and cell culture isolation. The results indicated that the CVA16-specific PCR was able to detect 0.03 50% tissue culture infective dose ($TCID_{50}$) of CVA16 per 100 μl (Table 4).

We have thus successfully developed a molecular assay for the specific identification of CVA16 directly in clinical specimens, cell culture material, or suckling mouse torso samples. Serotype-specific PCR assays have previously been described only for poliovirus serotypes 1 to 3 (9), EV70 (16), and EV71 (1), and most have not been evaluated for direct testing of clinical specimens. There are several potential advantages of typing enteroviruses by PCR rather than by serological methods. Primers are cheaper to produce than monoclonal or polyclonal antisera. Serotyping by neutralization is technically difficult, labor-intensive and, for CVA strains, may require the use of suckling mice. Furthermore, PCR is more rapid and amenable to standardization and offers potentially greater sensitivity, enabling typing to be achieved directly from clinical specimens.

Specificity was achieved in our assay provided that both primer sets were used. The enterovirus serotypes most closely related to CVA16, i.e., CVA2 to CVA5, CVA7, CVA8, CVA14, and EV71 (8), tested negative. However, in some instances, only prototype strains were available, and it will be necessary to further validate assay specificity using newer strains as they appear in clinical cases.

The CVA16-specific PCR will be useful for testing clinical specimens from cases of suspected CVA16 infections (such as HFMD) which test positive in generic enterovirus-reactive PCR assays and for typing cell culture or suckling mouse iso-

lates. When combined with nucleotide sequencing of PCR products, the CVA16-specific PCR may also be useful for studying the molecular epidemiology and evolution of CVA16.

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