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Variability in Molecular Typing of Coxsackie A Viruses by RFLP Analysis and Sequencing

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The aim of the present study was to develop an assay capable of classifying the Coxsackie A virus (CAV) prototype strains on the basis of restriction fragment length polymorphism (RFLP) analysis of 5′-UTR-derived reverse transcription polymerase chain reaction (RT-PCR) amplicons, and to determine how these data could be used for typing wild-type CAV isolates. Moreover, sequencing of the amplified genomic fragments of the clinical isolates, and comparison with all the published sequences of the respective genomic region of enterovirus reference and wild-type strains were attempted for typing of the isolates. Twenty-four prototype CAV strains from the 23 currently recognized serotypes were studied; most of them were successfully differentiated with the aid of four restriction endonucleases: HaeIII, HpaII, DdeI, and StyI. It was not possible to differentiate between CAV5, 7, and 16, or between CAV15 and 18 in this way, but the members of each of these two groups were satisfactorily differentiated with the aid of single-strand conformational polymorphism (SSCP) analysis of their RT-PCR amplicons. Fifteen clinical isolates, 13 of them of known CAV serotype, were also studied with the same four endonucleases and the results were compared with the data obtained from the RFLP analysis of the reference strains. The experimental results showed that only two clinical samples of previously known identity had an identical restriction pattern with the respective prototype strains. The sequences of the amplicons of the clinical isolates had the greatest percentage of alignment with enterovirus strains of a different serotype, indicating variability in the 5′-UTR and the inability to use the whole sequence of the amplicons for typing CAVs. The significance of the findings in relation to the possible usefulness of the RFLP-based method is discussed. J. Clin. Lab. Anal. 16:59–69, 2002.

Key words: Coxsackie A viruses; 5′-UTR; RT-PCR/RFLP; sequencing

INTRODUCTION

Coxsackie A viruses (CAVs), along with coxsackie B viruses, polioviruses, echoviruses, and enteroviruses 68–71, belong to the *Enterovirus* genus, one of the nine different genera that comprise the *Picornaviridae* family and the most important in terms of human pathogenicity. Twenty-three different antigenic types of CAVs are known (1–22 and 24, as type 23 was found to be indistinguishable from echovirus 9). Most CAV infections are asymptomatic, as is the case with all enteroviruses, which initially rendered this group of viruses unworthy of sustained investigation. As a consequence, knowledge of the diseases caused by this group of viruses still may be rather incomplete (1). Nevertheless, these viruses are known to be responsible for diseases such as herpangina (several serotypes), meningitis and encephalitis (often CAV7 and CAV9), and paralytic disease (outbreaks of CAV7 have been described) (2).

Despite the clinical significance of CAVs, they have received little attention, which is largely due to the practical difficulties in isolating, propagating and identifying these viruses. Initially, most CAV serotypes could only be propagated in suckling mice. To avoid this cumbersome procedure and improve the efficiency of virus isolation, several cell lines with improved characteristics for CAV susceptibility have been introduced. The World Health Organization recommends the use of seroneutralization pools of equine, mixed hyperimmune antisera, following isolation in cell culture, for the typing of some enteroviruses. The Lim Benyesh-Melnick

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(LBM) seroneutralization pools include antisera against 19 CAV serotypes, but the pools developed by the RIVM institute in Holland, which are steadily replacing the LBM pools, are able to detect CAV9 only. Moreover, typing by seroneutralization may frequently fail due to the fact that it is a labor-intensive, time-consuming procedure, and to the problem of "untypable" isolates. The high evolution rates of the antigenic sites result in the appearance of so-called "prime" strains, which consist of an antigenic continuum with known serotypes, and which cannot be typed with the available antisera. Several alternative methods have been presented for the identification of CAVs and other enteroviruses. Those methods rely on the use of fluorescent antibodies directly on specimen material, enzyme immunoassays with type-specific antisera (3), immunoelectron microscopy with polyvalent and type-specific antisera (4), and monoclonal antibodies against group-reactive (5) or type-specific epitopes (6) on the virus capsid surface. However, these methods have reduced specificity (e.g., Ref. 7) and they are time-consuming, since they rely on cell culture amplification of CAVs. Because of the drawbacks of methods based on cell culture and/or immunological detection, there is a growing tendency to use genetic information for the characterization of viruses and microorganisms in general. Spot hybridization using cDNA probes representing several different enterovirus subgroups has a limited diagnostic value in rapid, accurate detection and identification of enteroviruses from clinical material since its sensitivity with actual clinical specimens is only 33% or less (6). Oligonucleotide fingerprint analysis is another sensitive molecular method for the identification of CAVs which has been used in clinical and epidemiological cases of CAV infection (8,9). However, this technique is complex and technically demanding.

Numerous reverse transcription-polymerase chain reaction (RT-PCR) assays have been applied to RNA detection of most, if not all of the enterovirus serotypes, in an attempt to improve speed, sensitivity, and specificity (10,11). These assays were based on the detection of extremely conserved genomic sequences among the different serotypes, such as the 5′-untranslated region (5′-UTR), and provided new depth in virological research for both systematics and diagnostics. However, a limitation of most RT-PCR methods described so far is their inability to provide information on the serotype or other subclassification of enteroviruses. Therefore, it is necessary to supplement RT-PCR with methods to assess sequence differences in the PCR products, such as restriction fragment length polymorphism (RFLP) analysis (12–16), hybridization with type-specific probes (17), or single-strand conformational polymorphism (SSCP) (18). Nucleotide sequences of RT-PCR products would also be quite helpful—at least for research concerning the evolution and epidemiology of the viruses (although not for routine diagnosis of clinical isolates).

The aim of the research described here was the development of an assay that would allow detection and differential identification of the various reference strains of CAVs, and the evaluation of the possible usefulness of this method for identifying wild-type CAV isolates. The method was based on RFLP analysis of RT-PCR amplicons with four different restriction endonucleases. The genomic region of study was chosen from the highly conserved 5′-UTR in an attempt to reduce intratypic variation and to allow the detection of any intertypic differences in the studied region of the genome. The investigation was carried out on 24 different CAV reference strains from the 23 serotypes and on 15 clinical isolates. The sequences of the RT-PCR amplicons of most of the clinical isolates were also determined and were compared with all the available enterovirus sequences of the respective genomic region, which provided an indication of the suitability of 5′-UTR for typing of wild-type isolates. The possible diagnostic and epidemiological relevance of the results is discussed.

MATERIALS AND METHODS

Reference Strains

The 24 CAV reference strains from the 23 serotypes used in this study were kindly provided by the National Institute for Public Health and the Environment in Holland and by the Pasteur Institute in Paris, and are shown in Table 1.

Clinical Samples

Fifteen clinical strains were used in this study, the details of which are summarized in Table 2. Ten of these strains were in cell culture stock and recultured in RD cells, and five strains were isolated from stool samples during routine diagnosis at the Hellenic Pasteur Institute. Two grams of each stool sample were added to a suspension containing 10 ml phosphate-buffered saline (PBS), 5 g of glass beads, and 0.5 ml chloroform. Following centrifugation at 3,000 rpm for 30 min at +4°C, the supernatant was removed and used for the inoculation of the cell cultures (19).

Cell Cultures

The cell line rhabdomyosarcoma (RD) was used in cell culture tubes (Becton Dickinson, Franklin Lakes, NJ) containing 2 ml of D-MEM, and 400 ml of inoculum (inoculated cell culture, or processed stool) were added to each tube. The inoculated tubes were then incubated in a roller at 37°C for a period of 1–7 days, until a complete cytopathic effect (CPE) was observed under an ordinary light microscope. Uninfected RD cells were used as negative controls.

RNA Extraction

When a complete CPE was observed, the infected cells were frozen at –80°C and thawed three times; 350 μl of the cell culture were used for RNA extraction with the phenol-based TRIzol commercial kit by Gibco BRL (Life Technologies Ltd., Paisley, UK) according to the manufacturer's instructions.

TABLE 1. Details of the CAV reference strains used in the present study

Virus	Strain	Year of isolation	Illness	Source	Type of material used
Coxsackie A1	Tompkins	1947	Poliomyelitis[a]	RIVM	SM[d]
Coxsackie A2	Fleetwood	1947	Poliomyelitis[a]	RIVM	RD[e]
Coxsackie A3	Olson	1948	Aseptic Meningitis	RIVM	RD[e]
Coxsackie A4	High Point	1950	Isolated from sewage of polio community	RIVM	RD[e]
Coxsackie A5	G.S.	1950	Poliomyelitis	HPI[b]	RD[e]
Coxsackie A6	C.G.	1949	Aseptic Meningitis	HPI[b]	RD[e]
Coxsackie A7	W. Parker	1949	Aseptic Meningitis	RIVM	GaBi[e]
Coxsackie A8	Donovan	1949	Poliomyelitis	RIVM	GaBi[e]
Coxsackie A9	P. Bozek	1950	Aseptic Meningitis	RIVM	BGM[e]
Coxsackie A9	Griggs	Unknown	Unknown	IPP[c]	RD[e]
Coxsackie A10	Kowalik	1950	Aseptic Meningitis	RIVM	RD[e]
Coxsackie A11	Belgium-1	1951	Epidemic myalgia	IPP[c]	RD[e]
Coxsackie A12	Texas-12	1948	Isolated from flies in polio community	RIVM	RD[e]
Coxsackie A13	Flores	1952	None	RIVM	GaBi[e]
Coxsackie A14	G-14	1950	None	RIVM	RD[e]
Coxsackie A15	G-9	1950	None	RIVM	GaBi[e]
Coxsackie A16	G-10	1951	None	HPI[b]	RD[e]
Coxsackie A17	G-12	1951	None	RIVM	GaBi[e]
Coxsackie A18	G-13	1950	None	RIVM	GaBi[e]
Coxsackie A19	Dohi	1952	Guillian-Barré syndrome	RIVM	SM[d]
Coxsackie A20	IH-35	1955	Infectious hepatitis	RIVM	GaBi[e]
Coxsackie A21	Coe	Unknown	Mild respiratory disease	RIVM	GaBi[e]
Coxsackie A22	Chulman	1955	Vommiting & diarrhea	RIVM	SM[d]
Coxsackie A24	Joseph	1952	None	RIVM	HEL[e]

The information about year of isolation and the illness originally associated with each strain was obtained from Pulli et al. (26).
[a]Isolated from cases of dual infection with polioviruses, which were presumably responsible for the paralytic illness.
[b]Hellenic Pasteur Institute.
[c]Institute Pasteur, Paris.
[d]Virus propagated in suckling mouse material, which was used directly for RNA extraction.
[e]Cell lines in which the viruses that were used for cell culture inoculation had been previously been propagated. RD, rhabdomyosarcoma cells; HEL, human embryonic lung fibroblasts; GaBi, HEL-type cells; BGM, buffalo green monkey kidney cells.

RT-PCR

The primers UC_{53} (anti-sense, with the sequence 5′-TTGTCACCATAACCAGCCA-3′) and UG_{52} (sense, with the sequence 5′-CAAGCACTTCTGTTTCCCCGG-3′), which were used for the enterovirus-specific RT-PCR, were selected so as to be homologous to respective parts within the highly conserved 5′-UTR region. They were purchased from Genosys Biotechnologies, Europe (Cambridge, UK). The antisense primer is one base shorter than the primer 3 described by Zoll et al. (20), whereas the sense primer is precisely the same as the primer 1 used by the same authors. The relative position of the target sequences of these primers on the genome of enteroviruses with known sequences has been shown previously (21). These primers yield amplicons approximately 435 bp long; they were adjusted to a concentration of 20 pmol/μl in sterile, RNAse-free distilled water (Sigma Aldrich, St. Louis, MO) and were stored at –20°C. The reverse transcription reaction for the conversion of the isolated RNA into cDNA, and subsequent amplification of the cDNA were carried out as previously described (22). Ten μl of each amplified product was analyzed by agarose gel electrophoresis in 2.5% agarose (ultrapure agarose, electrophoresis grade; Gibco BRL) containing 1 μg/ml ethidium bromide in Tris-boric acid-EDTA (TBE) buffer. The amplicons were then visualized through a UV transilluminator FOTO/PHORESIS I, FOTODYNE (Hartland, WI).

RFLP Analysis

Twenty microliters of the amplicons were studied with the following restriction enzymes: HpaII, DdeI (New England Biolabs, Beverly, MA), HaeIII and StyI (Promega Corporation, Madison, WI). Twenty units from each restriction enzyme; the appropriate buffer; and distilled, DNAse/RNAse-free sterile water (Sigma Aldrich, St. Louis, MO) were added to each sample to a final volume of 30 μl. The samples were then incubated at 37°C for 2 hr and the products were subjected to electrophoresis in 3% gels made from high-resolution agarose (Metaphor FMC Bioproducts, Rockland, ME), containing 1μg/ml ethidium bromide. They were then visualized through a UV transilluminator. The molecular weight of the restriction fragments was determined with the aid of GelPro Analyzer software (Version 3.0, Media Cybernetics).

TABLE 2. Details of the CAV clinical samples that were used in the present study

Serotype	Source	Illness	Designation
CAV9	University of Essex	Unknown	A
CAV9	University of Essex	Unknown	B
CAV9	University of Essex	Unknown	C
CAV9	University of Essex	Unknown	D
CAV9	University of Essex	Unknown	E
CAV9	Cantacuzino Institute, Romania	Transient spinal paralysis	F
CAV9	Cantacuzino Institute, Romania	Persistent spinal paralysis	G
CAV9	Cantacuzino Institute, Romania	Transient spinal paralysis	H
CAV8	Cantacuzino Institute, Romania	No clinical signs	I
CAV16	Hellenic Pasteur Institute	HFMD[a]	J
CAV16	Hellenic Pasteur Institute	HFMD[a]	K
CAV16	Hellenic Pasteur Institute	HFMD[a]	L
CAV4	Cantacuzino Institute, Romania	Meningitis	M
Unidentified	Hellenic Pasteur Institute	Diarrhea	N
Unidentified	Hellenic Pasteur Institute	Meningitis	O

[a]Hand-foot-and-mouth disease.

SSCP

SSCP analysis of the PCR amplicons was carried out to differentiate between those reference strains (CAV5-CAV7-CAV16 and CAV15-CAV18) that could not be satisfactorily differentiated by RFLP analysis alone. Under nondenaturing conditions, single-stranded DNA has a folded conformation that is determined by intrastrand complementarity and, hence, by its sequence. In the specific SSCP analysis, 5 μl of the PCR products of the different viral strains were added to 20 μl of SSCP buffer (95% formamide and 5% bromophenol blue) and converted into single-stranded molecules by heating them at 95°C for 5 min and cooling them immediately on ice. The amplicons were then subjected to vertical electrophoresis in a polyacrylamide gel (49/1 acrylamide/bis) at low temperature (12 ± 1°C), in an attempt to increase the resolution efficiency of the gel. The single-stranded PCR products were then visualized by treating the polyacrylamide gel with silver staining using the commercial kit GelCode™ (Pierce, Rockford, IL). The difference in sequences of the PCR amplicons of different viruses is generally detected by the corresponding differential mobility of these single-stranded amplicons. The sensitivity of SSCP tends to decrease with increasing fragment length (23). It has been reported that it detects >90% of all single-base substitutions in 200-nucleotide fragments and >80% in 400-nucleotide fragments (23). This led to the use of the primers P_3 (antisense, with the sequence 5′-ATTGTCACCATAAGCAGCCA-3′, i.e., the same with UC_{53} but only one base longer) and P_2 (sense, with the sequence 5′-TCCTCCGGCCCCTGAATGCG-3′), as originally used by Zoll et al. (20) (primers 3 and 2, respectively), for the production of 155-bp-long amplicons that were analyzed by SSCP. The relative position of the target sequences of these primers on the genome of enteroviruses with known sequences, according to the picornavirus sequence database, has already been described (21).

Sequencing

Since the eight CAV9 and three CAV16 clinical isolates did not have an identical restriction profile with the respective prototype strains, the UC_{53}/UG_{52}-produced RT-PCR amplicons of these isolates were sequenced and compared with the sequences of the respective genomic regions of all the reference and wild-type enterovirus strains, for which such data exist in the Picornavirus Sequence Database (http://www.iah.bbsrc.ac.uk/virus/Picornaviridae/SequenceDatabase), with the aid of ClustalW (version 3.00) computer software, obtained online from the website of the Pasteur Institute in Paris (http://www.bioweb.pasteur.fr/#log). The UC53/UG52-produced amplicons of isolates "N" and "O" were also sequenced and compared with the respective sequences of the rest of the enteroviruses, in an attempt to extract more information regarding the identity of these isolates. Fifty microliters of the RT-PCR amplicons of all the clinical isolates were run in a 1% low melting agarose gel (Metaphor FMC Bioproducts, Rockland, ME) containing 1 μg/ml ethidium bromide. The DNA bands corresponding to the PCR products were excised from the gel and transferred to clean, 1.5-ml Eppendorf tubes. They were then purified with the aid of the QIAquick gel extraction kit (Qiagen, Inc., Chatsworth, CA) and suspended in 10 μl of RNAse-free, sterile, distilled water. The sequencing reaction was carried out according to the ABI PRISM™, Big Dye™ Terminator Cycle Sequencing ready reaction kit protocol (PE Applied Biosystems). Both strands of the purified DNA were sequenced using the two enterovirus-specific primers UC_{53} and UG_{52}, respectively. A 0.2-mm-thick polyacrylamide gel was used for the electrophoresis of the sequencing products. The electrophoresis and the differential detection of the sequencing products were carried out with the ABI PRISM 377 DNA Sequencer (Perkin Elmer Corp.).

RESULTS

Cell Culture Isolation

All prototype and wild-type strains used in this study were successfully grown in RD cells. In most cases several passages were needed to obtain a satisfactory CPE, especially during virus isolation from stool samples, despite the initial high titer of the inoculum from the stock reference strains, confirming the difficulty of growing CAVs in cell cultures.

RT-PCR

The primers UC_{53}/UG_{52} used in this study were successful in the detection of all the 24 reference and 15 wild-type CAV

strains within a few hours after the observation of a complete CPE. This was in contrast with the results of Zoll et al. (20), who did not obtain an amplification product for CAV11, 17, and 24 with a similar set of primers targeting the same genomic regions. The amplicons produced were approximately 435 bp long, as was the case for other enteroviruses detected by the same set of primers (20).

RFLP Analysis of the UC_{53}/UG_{52}-Produced RT-PCR Amplicons of the Prototype Strains

The combination of four restriction endonucleases allowed the intertypic differentiation of the prototype strains, with the exception of CAV5-CAV7-CAV16 and CAV15-CAV18; the members of each of these groups had identical restriction patterns between them. Table 3 shows the collective data of the RFLP analysis for all the 24 prototype strains in terms of number and length of the restriction fragments. The groups of reference strains with the same restriction profile for each endonuclease are also shown. Most of the prototype strains were differentiated with the restriction enzyme DdeI only, apart from four groups (first group: CAV2, 3, 5, 7, 8, and 16; second group: CAV4, 8, 9 "Griggs," 9 "P. Bozek," and 14; third group: CAV11, 15, 18, and 24; and fourth group: CAV13, 20, and 21) (Table 3). The members these groups were further differentiated with the restriction enzymes HaeIII, HpaII, and StyI, with the exception of CAV5, 7, 15, 16, and 18, which were differentiated by SSCP analysis. For this reason we propose a flow chart for the identification of CAV reference strains with the four restriction enzymes that were used in the present study: DdeI, HaeIII, HpaII, and StyI (Fig. 1). Slight statistical variation in the results of the analysis of the RFLP data by GelPro Analyzer software for those strains which had an otherwise identical restriction pattern led to the establishment of certain criteria by which the comparison of the numeric data concerning the length of the restriction fragments would determine the prototype strain groups shown in Table 3. Specifically, a difference of more than 10 bases in length for restriction fragments larger than 200 bp was considered to yield a discriminatory restriction pattern, whereas for fragments smaller than 200 bp a difference of more than five bases in length was considered to be large enough to discriminate between different strains.

The restriction pattern of the known sequences of the 5′-UTR-derived genomic fragments of the CAV reference strains, which are available from the GenBank sequence database, as produced by the four different restriction endonucleases, was simulated with the aid of the Gene Runner computer software (version 3.00, Hastings Software Inc.; software obtained on-line from www.generunner.com) and compared with the results of the practical application of the RFLP analysis of the RT-PCR amplicons. The results verified the experimental data obtained from the RFLP analysis of the amplicons (data not shown).

TABLE 3. RFLP analysis with DdeI, HaeIII, StyI and HpaII of the UC_{53}/UG_{52}-produced RT-PCR amplicons of the reference strains and clinical isolates

Restriction enzyme	RFLP profile
DdeI Reference strains	
CAV2, 3, 5, 7, 8, 16	307+128
CAV10	307+94+34
CAV4, 6, 9[a], 9[b], 14	307+105+23
CAV1, 11, 15, 18, 22, 24	285+125+25
CAV12	412+23
CAV13, 19, 20, 21	285+150
CAV17	No cut
Clinical samples	
A, B, C, D, F, H, I	307+128
E	No cut
G, J, K, L, M	307+105+23
N, O	341+71+23
HaeIII Reference strains	
CAV1, 19	154+147+80+36+18
CAV2, 4	146+132+81+76
CAV3	154+145+81+55
CAV5, 6, 7, 8, 10, 12, 14, 15, 16, 18, 20, 24	208+146+81
CAV9[a], CAV9[b]	146+81+78+76+54
CAV13	191+146+56+24+18
CAV17, 11	209+145+70+11
CAV21, 22	190+147+80+18
Clinical samples	
A, B, C, D, H, I, J, K, L	208+146+81
E, G	154+146+81+54
M, N, O	144+129+80+75
F	186+146+81+31
StyI Reference strains	
CAV1	214+123+86+12
CAV2, 3, 5, 7, 9[a], 9[b], 14, 15, 16, 18	No cut
CAV4, 6, 12	214+209+12
CAV8, 11, 17, 24	334+101
CAV10, 19, 22	209+113+101+12
CAV13	313+122
CAV20	212+122+101
CAV21	221+113+101
Clinical samples	
A, C, D, F, H, J, K, L, M, N, O	214+209+10
B, E	No cut
G	209+110+104
I	332+102
HpaII Reference strains	
CAV2, 3, 4, 5, 6, 7, 8, 9[a], 9[b], 10, 12, 14, 16	213+149+55+18 (Cluster II)
CAV1, 13, 15, 17, 18, 19, 20, 21, 24	161+148+108+18 (Cluster I)
CAV11, 22	148+121+108+40+18 (Cluster I)
Clinical samples	
A, B, C, D, E, F, G, H, I, J, K, L, M, N, O	213+149+55+18 (Cluster II)

The sub-classification of the serotypes into the two 5´-UTR genetic clusters (Cluster I for "polio-like" cluster and Cluster II for "CBV-like" cluster) with the restriction enzyme HpaII is also shown.
[a]Reference Strain P. Bozek.
[b]Reference Strain Griggs.

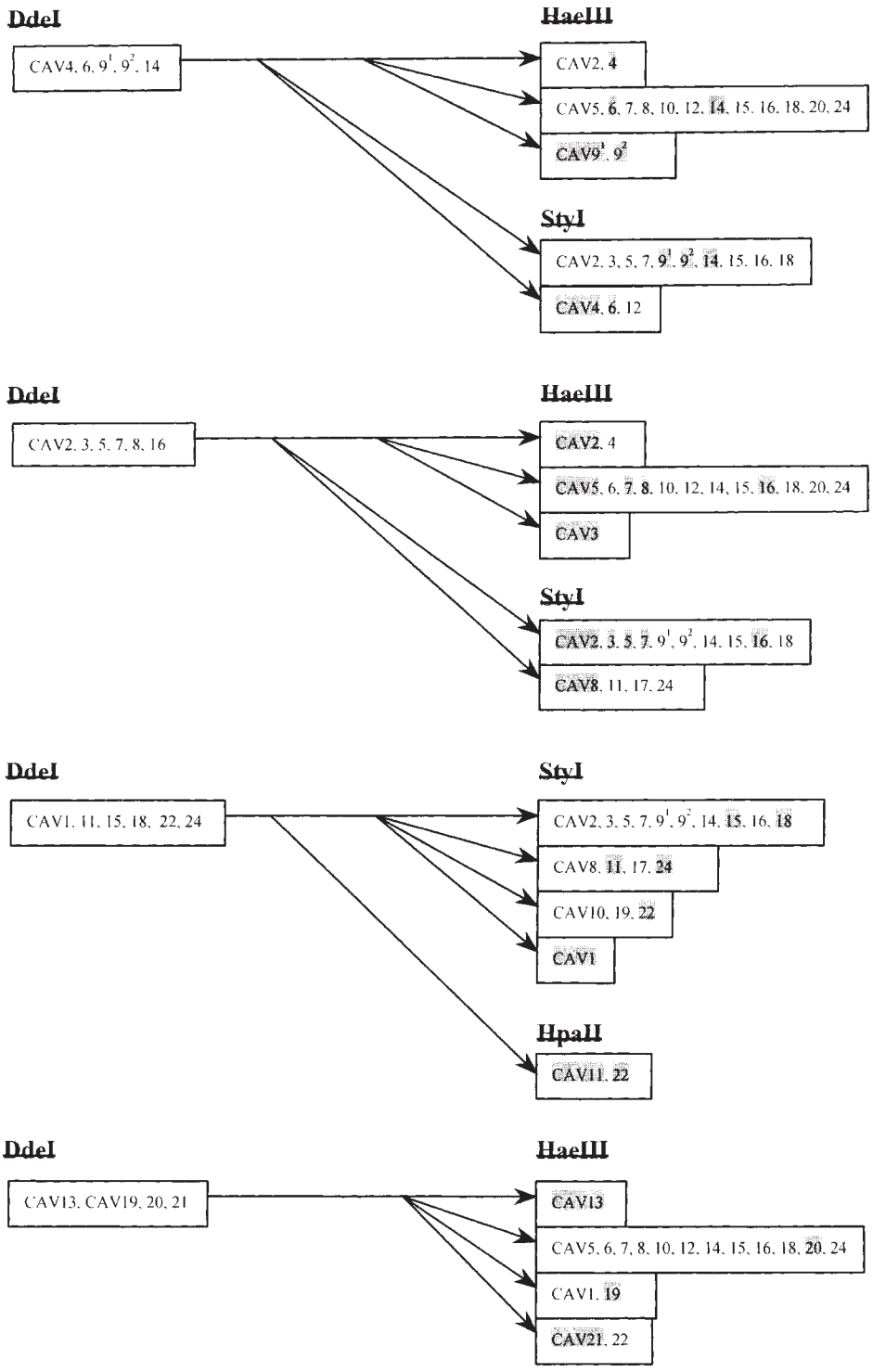

Fig. 1. Flowchart for the differentiation of the prototype strains that could not be identified with the restriction enzyme DdeI alone. The prototype strains were further differentiated with the restriction enzymes HaeIII, HpaII, and StyI, with the exception of strains CAV5-7-16 and CAV15-18. The DdeI group members are highlighted in each of the other enzyme groups. [1]Reference strain "P. Bozek." [2]Reference strain "Griggs."

SSCP Analysis of the P_3/P_2 Produced RT-PCR Amplicons

The prototype strains (CAV5-7-16 and CAV15-18) that could not be differentiated with the restriction endonucleases were successfully differentiated by SSCP analysis. Figure 2a shows the results of the SSCP analysis of the P_3/P_2-produced RT-PCR products for CAV5, 7, and 16, and Fig. 2b shows the respective results for CAV15 and 18.

RFLP Analysis of the UC_{53}/UG_{52}-Produced RT-PCR Amplicons of the Clinical Isolates, and Comparison of Their Restriction Pattern With That of the Reference Strains

Table 3 shows the collective data of the RFLP analysis for all the clinical enteroviral isolates in terms of number and length of the restriction fragments. Only two clinical samples (samples "I" and "M") had an identical restriction pattern with the respective prototype strains with all the restriction enzymes used, whereas inconclusive data was obtained for the typing of the rest of the clinical isolates, as shown in Table 4.

Overall, there is no clear relationship between the nature of the 5′-UTR, as revealed by RFLP analysis, and the serotype of clinical isolates. For instance, none of the eight CAV9 isolates gave a pattern equivalent to the reference strains "P. Bozek" and "Griggs" when the amplicons were cut with HaeIII. Only one of the eight DdeI digests was equivalent to the prototypes (sample "G"), and only two isolates ("B" and "E") shared the lack of restriction site for StyI exhibited by the prototypes. Thus, apart from digestion with HpaII, these CAV9 strains showed few features with CAV9 prototypes. The best match (samples "B," "E," and "G") showed two out of four identical enzyme digests with the prototypes, while the rest showed only one out of four (HpaII). In contrast, four out of nine isolates (A, C, D, H) exhibited identical patterns with all four enzymes.

Two of the clinical isolates, "N" and "O," had not been serotyped previously. Both gave an identical restriction profile with prototype strain CAV4 "High Point" using three out of four restriction enzymes. To investigate this further, the sequences of the amplicons from the isolates and from CAV4 "High Point" were determined (data not shown). Both had a 90% alignment with CAV4 "High Point." However, they had a greater identity with other reference strains, achieving the best score of alignment (95%) with swine vesicular disease virus (SVDV). The failure to identify many of the clinical isolates and the samples "N" and "O" indicated that 5′-UTR is so variable that the use of the specific restriction endonucleases may not be sufficient for the correct typing of clinical isolates.

Enterovirus Subgrouping by RFLP Analysis With the Restriction Endonuclease HpaII

Although serotypes of clinical samples could not be predicted from 5′-UTR RFLPs, it is possible that these could be used for subgrouping CAVs. HpaII digests predicted the first

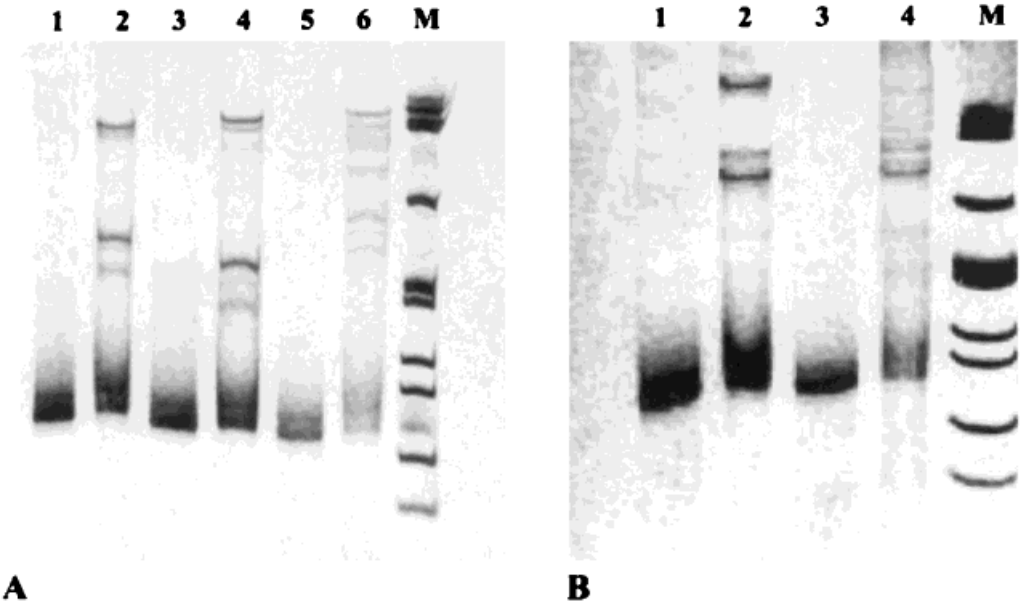

Fig. 2. The results of SSCP analysis of single-stranded P_3/P_2-produced RT-PCR amplicons of (**a**) CAV5, CAV7, and CAV16, and (**b**) CAV15 and CAV18, for which no satisfactory differentiation with the five restriction endonucleases was achieved. a: Lanes 2, 4, and 6 show the differential electrophoretic mobility of the conformers corresponding to CAV16, CAV7, and CAV5, respectively, whereas lanes 1, 3, and 5 show the nondenatured, double-stranded RT-PCR amplicons. b: Lanes 2 and 4 show the differential electrophoretic mobility of the conformers corresponding to CAV18 and CAV15, respectively, whereas lanes 1 and 3 show the nondenatured, double-stranded RT-PCR amplicons. In both figures M shows the molecular weight marker (φχ174 RF DNA/HaeIII fragments (Gibco BRL)).

TABLE 4. Identity of clinical isolates as inferred by RFLP analysis and sequencing

Sample	Serotypic identity	Identity inferred by RFLP analysis	Enterovirus strain with best score of sequence alignment (percentage)
A	CAV9	CAV5, 7, 8, 16[b]	ECV8 "Bryson" (92%)
B	CAV9	CAV5, 7, 16[c]	CAV9 "Griggs," CAV16 "G-10," CAV16 "Tainan," CBV1 "Japan," CBV2 "Ohio-1," ECV4 "Pesacek," ECV8 "Bryson,"[d] ECV9 "Hill," (92%)
C	CAV9	CAV5, 7, 8, 16[b]	ECV25 "Th222" (91%)
D	CAV9	CAV5, 7, 8, 16[b]	ECV25 "Th222" (91%)
E	CVA9	CAV3[b]	ECV3 "Morrissey," ECV8 "Bryson,"[d] ECV25 "JV-4" (92%)
F	CAV9	CAV2, 3, 5, 7, 8, 16[a]	CBV5 "Faulkner" (90%)
G	CAV9	CAV4, 6, 9, 14[a]	CBV5 "Faulkner" (93%)
H	CAV9	CAV5, 7, 8, 16[b]	CBV5 "Faulkner" (92%)
I	CAV8	CAV8[c]	—[e]
J	CAV16	CAV6[b]	CAV16 "Tainan" (96%)
K	CAV16	CAV6[b]	CAV16 "Tainan" (96%)
L	CAV16	CAV6[b]	CAV16 "Tainan" (96%)
M	CAV4	CAV4[c]	—[e]
N	Unknown	CAV4[b]	SVDV "UKG/27/72" (95%)
O	Unknown	CAV4[b]	SVDV "UKG/27/72" (95%)

[a]Best match with 2 restriction enzymes.
[b]Best match with 3 restriction enzymes.
[c]Best match with all the restriction enzymes.
[d]ECV8 is considered to be serotypically identical with ECV1.
[e]Not sequenced.

5′-UTR group accurately. With the exception of CAV11, the entire first cluster of viruses gave fragments of approximately 160, 148, and 108 bp (Table 2). These are due to an HpaII site at genome positions 342 and 449 in one member of the cluster, CAV21. The second cluster members gave bands of 213, 149, and 55 bp, due to sites at positions 235 and 448 in CAV9, a typical member of this cluster. CAV11 is a member of the first cluster, but gives four bands with HpaII: 148, 121, 108, and 40 bp. This corresponds to the characteristic pattern given by the first-cluster members, except that the 160-bp fragment presumably split by an extra HpaII site. All of the serotyped clinical isolates used represent serotypes in the second genetic cluster and, as expected, all HpaII digests (and those of the viruses of unknown serotype) gave bands characteristic of this cluster as well (Table 2). This confirms that RFLP analysis can be used to ascribe CAVs to 5′-UTR clusters.

Analysis of Sequence Data

The comparison of the sequences of the CAV clinical isolates with the sequences of the same 5′-UTR fragment of the rest of the enteroviruses verified that the 5′-UTR of the CAVs and all the enteroviruses is highly conserved, as is generally known (24–27). Enteroviruses are classified into two clusters on the basis of 5′-UTR; one cluster includes all the polioviruses; coxsackieviruses A11, 13, 15, 17, 18, 21, and 24; and enterovirus (EV) 70; whereas the second cluster contains the CBVs, echoviruses, and the rest of the CAVs and EV71 (24–26) (Siafakas et al., unpublished). The analysis of the data led to the classification of all the clinical isolates to the second cluster, as was expected from their already-known serotype, confirming the subgrouping by RFLP analysis with HpaII.

The restriction profile of the 5′-UTR-derived sequences of the clinical isolates, as produced by the four different restriction endonucleases, was simulated with the aid of Gene Runner software and compared with the results of the RFLP analysis of the RT-PCR amplicons. The results verified the experimental data obtained from the RFLP analysis of the amplicons (data not shown).

The analysis of the sequence data also showed that the sequences of the amplicons of most of the clinical isolates had a greater percentage of alignment with prototype strains of a different enterovirus serotype, as shown in Table 4, indicating the presence of variability and the inability to use the whole sequence of the amplicons for typing CAVs. For instance, CAV9 isolate "A" had a greater percentage of alignment (92%) with ECV8 reference strain "Bryson," whereas the sequence of CAV9 isolates "C" and "D" were best aligned with ECV25 strain "Th222." Only the three CAV16 isolates (samples "J," "K," and "L") had the highest score of alignment with the CAV16 strain "Tainan" and the second best score (93%) with CAV16 prototype strain "G-10." There was even one case, CAV9 sample "B," in which the greatest score of alignment (92%) was achieved with CAV9 reference strain "Griggs," but also with seven other enterovirus strains of a different serotype.

DISCUSSION

In this study, the broadly reactive for enteroviruses primers UC_{53}/UG_{52} and P_3/P_2 primer pairs were used. With the

former set of primers it was possible to detect all the 24 prototype CAV strains and the 15 clinical isolates used in this study. The RT-PCR assay was supplemented with the two molecular genotyping methods (RFLP and SSCP) described in this study, in an attempt to determine a rapid and reliable means for the subclassification of clinical CAV isolates.

RFLP analysis of RT-PCR amplicons has been suggested and used in the past for the rapid differentiation of enterovirus serotypes and for the demonstration of genomic variability within the enterovirus genome (12,14,16). In the present study, four different restriction endonucleases were used in an attempt to optimize the detection of intertypic genetic differences amongst the different serotypes. The results of the RFLP analysis led to a satisfactory genetic discrimination of the prototype strains with four restriction enzymes (HaeIII, HpaII, DdeI, StyI), with the exception of CAV5-7-16 and CAV15-18. The results support the possible usefulness of the RT-PCR/RFLP-based methodology for the differential identification of prototype strains, and for the extension of this method to clinical and epidemiological practice. Nevertheless, despite the possible benefits of RFLP analysis for correct typing of CAVs, only two clinical samples ("I" and "M") had restriction patterns identical to the respective prototype strains of the four restriction enzymes that were included in this study (Table 4). Furthermore, there is the possibility that the actual sequence of a previously unidentified clinical isolate, typed as a specific strain with the restriction endonucleases, may differ significantly from that of the respective prototype strain with which it had an identical restriction profile. This is a result of the restriction enzymes identifying only a limited part of the genomic area of study. Such an example was the case of samples "N" and "O," which were typed as CAV4 with three out of four enzymes (Table 4). Sequencing of these samples revealed that their amplicons had a 90% alignment with the respective prototype strain and a greater percentage of alignment (95%) with the respective genomic region of SVDV.

Sequencing of the UC_{53}/UG_{52}-produced RT-PCR amplicons also produced highly variable results (with the exception of the three CAV16 isolates) when the isolates were compared with their respective prototype strains, since the sequence differences that were observed could not be classified as monophyletic within a single serotype. Therefore, the experimental results clearly showed the existence of genetic variability in the 5′-UTR, which led to the inability to type most of the clinical samples. Inherent to the RNA genome of enteroviruses and the lack of proofreading activity of the virus-encoded RNA polymerase is its highly mutable nature. This is due to point mutations, intra- or interspecific recombination events with members of the same group of viruses or with members from a different group, respectively, or even possible recombination with genetic material of cellular origin (27). For this reason, enteroviruses exist in a form of "quasispecies" populations, i.e., members of the same species in the same population are not completely identical. It is possible that all of these factors contributed to the observed variability of the 5′-UTR and, thus, to the failure to type the clinical isolates with the four restriction enzymes.

Using RFLP analysis, Kuan (15) studied 297-bp-long fragments from the 5′-UTR of six different CAV reference serotypes produced by a nested PCR (n-PCR) assay, along with another 18 enteroviruses, and used these data to identify enterovirus-infected specimens in laboratory diagnosis. He used three different restriction enzymes (StyI (which was also used in this study), BglI, and XmnI). The 5′-UTR fragment he studied is part of the 435-bp-long fragment that was the subject of research in the present study. Kuan (15) found that 14 out of 16 human enterovirus-infected specimens exhibited restriction patterns identical to those of the corresponding prototypes, including the reference strains CAV2, 3, 5, 6, and 7. The system with the restriction enzymes presented here was unable to type wild-type isolates on the basis of the UC_{53}/UG_{52}-produced 5′-UTR fragment, which contains the fragment studied by Kuan (15), and showed the variability that would impair typing efforts of similar rationale and methodology. Therefore, the system proposed by Kuan (15) was also tested in the clinical isolates that were sequenced in this study by simulating and comparing (with the aid of the Gene Runner computer software) the restriction pattern on the sequences of both CAV9 and CAV16 prototype and clinical strains with the three restriction endonucleases used by Kuan (15). This simulation showed that the three different restriction enzymes he used were not able to type the wild-type isolates used in the present study. For instance, only two CAV9 clinical strains (CAV9 "F" and CAV9 "G") of the eight included in this study had an identical StyI-produced restriction pattern with CAV9 reference strains "Griggs" and "P. Bozek." Concerning XmnI, only two CAV9 clinical samples (CAV9 "F" and CAV9 "H") also had an identical restriction pattern with the CAV9 prototype strains, whereas with BglI, three CAV9 isolates (CAV9 "A," CAV9 "E," and CAV9 "F") showed the same profile with the respective prototype strains. Furthermore, even those samples which had an identical restriction pattern with the respective prototype strains using StyI, BglI, and XmnI had an identical restriction pattern with other prototype strains as well. For example, the three CAV16 isolates had an identical restriction profile with the respective prototype strain using the restriction enzyme BglI, but they also had the same restriction profile with CAV5, 6, 12, 13, 18, 20, and 21.

Even if RFLP analysis was complicated by mutation/recombination events, it would provide significant biological and epidemiological information by showing genetic variability between members of the same serotype, or between viruses with a varying degree of genetic relatedness between them. Siafakas et al. (21) used the same RT-PCR/RFLP method described in this study to show the epidemiological relatedness of 16 clinical cases of enteroviral

meningitis. However, it must be pointed out that this RT-PCR/RFLP-based typing method with the same four restriction enzymes plus AvaI has been successfully used to type polioviruses (13).

Those prototype strains (CAV5-7-16 and CAV15-18) which could not be differentiated with RFLP analysis were analyzed by the very sensitive method of SSCP analysis. This method was applied successfully for denatured PCR products from wild-type and mutant genomes by Hayashi (23), whereas Fujioka et al. (18) used SSCP of PCR products from the 5′-UTR using the same set of primers as the primer pair P_3/P_2 described in this study, for the analysis of genotypes of 14 enteroviruses, including CAV7 and 9. In the present study, CAV5, 7, and 16 reference strains, which could not be separately identified by RFLP analysis, had a different SSCP electrophoretic profile. The same result was obtained when CAV15 and CAV18 reference strains were compared. In general, the power of this technique for the detection of mutation and recombination events, with epidemiological and clinical value, was sustained. However, because of the power of this technique in identifying genomic fragments differing by only a few nucleotides, it is doubtful whether it could be used for the typing of wild-type isolates, due to the existence of intraserotypic genetic variability within the 5′-UTR. The diagnostic value of this method remains to be further assessed, although Siafakas et al. (21) drew useful conclusions concerning the epidemiological relationship between different isolates with this method during an outbreak of aseptic meningitis. Maisonneuve et al. (28) applied RT-PCR-SSCP to the study of 154-bp-long genomic fragments from the 5′-UTR of different echovirus 30 isolates, which were responsible for a meningitis outbreak in France. They managed to demonstrate the existence of two dominant clones of the serotype responsible for the epidemic.

In conclusion, this study attempted to identify 24 CAV reference strains from the 23 serotypes in order to provide further insight into the biology of this often neglected, but clinically important group of viruses, and the possibilities for their molecular typing. The highly conserved 5′-UTR was chosen in an attempt to use a broadly reactive for enteroviruses RT-PCR assay for their clinical isolation, and at the same time to exploit any genetic differences in that region amongst the different serotypes for their accurate and rapid identification during clinical and epidemiological applications. The differentiation of the reference strains was successful with the molecular techniques described here. However, the poor correlation between the 5′-UTR profile and serotype in clinical samples suggests that it is unlikely that this method can be used for the actual serotyping of CAV clinical isolates. Nevertheless, important information on grouping into 5′-UTR clusters can be obtained. It will be interesting to determine whether this can be extended to definition of the genotype to which isolates belong. These questions and their possible significance to the biology, pathogenesis, and epidemiology of CAVs remain to be further assessed.

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