Journal of General Virology (2000), **81**, 1361–1372. Printed in Great Britain

Molecular epidemiology and evolution of coxsackievirus A9

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Genetic relationships between 35 clinical isolates of coxsackievirus A9 (CAV9), collected during the last five decades from different geographical regions, were investigated by partial sequencing. Analysis of a 150 nucleotide sequence at the VP1/2A junction region identified 12 CAV9 genotypes. While most of the strains within each genotype showed geographical clustering, the analysis also provided evidence for long-range importation of virus strains. Phylogenetic analysis of a longer region around the VP1/2A junction (approximately 390 nucleotides) revealed that the designated genotypes actually represented phylogenetic lineages. The phylogenetic grouping pattern of the isolates in the analysis of the VP4/VP2 region was similar to that obtained in the VP1/2A region whereas analysis of the 3D region indicated a strikingly different grouping, which suggests that recombination events may occur in the region encoding the nonstructural proteins. Analysis of the deduced amino acid sequences of the VP1 polypeptide demonstrated that the RGD (arginine-glycine-aspartic acid) motif, implicated in the interaction of the virus with integrin, was fully conserved among the isolates.

Introduction

Human enteroviruses, small RNA viruses in the family *Picornaviridae*, are conventionally subgrouped into polioviruses (PVs; 3 serotypes), coxsackie A viruses (CAVs; 23 serotypes), coxsackie B viruses (CBVs; 6 serotypes), echoviruses (EVs; 28 serotypes) and enteroviruses 68–71, mainly on the basis of their pathogenicity in experimental animals. Division of coxsackieviruses, which are associated with a wide range of human diseases such as meningitis, encephalitis, paralysis, myocarditis, pleurodynia, exanthemas and respiratory illnesses, into A and B subgroups depends on the disease signs and histopathological lesions that occur after inoculation into newborn mice. A proposed new species classification of human enteroviruses is based on genetic relationships between the virus strains (Hyypiä *et al*., 1997; King *et al*., 1999). According to sequence homology in the coding and the 3′ noncoding region (NCR) of the approximately 7·5 kb ssRNA genome, enteroviruses can be divided into four clusters, A–D (Pöyry *et al*., 1994, 1996; Hyypiä *et al*., 1997); CAV16 and enterovirus 71 belong to cluster A, CAV9, CBVs and all sequenced EVs form cluster B, CAV21, CAV24 and PVs are found in cluster C while enterovirus 70 represents cluster D. In the 5′NCR, only two molecular groups are observed; group I includes cluster C and D viruses and group II cluster A and B viruses. Findings based on partial sequencing of different genomic regions have demonstrated that all enteroviruses can be similarly classified (Pulli *et al*., 1995; Huttunen *et al*., 1996; Oberste *et al*., 1998, 1999).

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The GenBank accession numbers of the sequences reported in this paper are AF166172–AF166253 and AF224645–AF224661.

Enteroviruses, like other RNA viruses, have a high mutation rate due to the lack of proofreading activity during genome replication. It has been estimated that approximately one mutation is generated per newly synthesized genome (Drake *et al*., 1993). In addition to mutations, recombination is involved in enterovirus evolution. Homologous recombination, which is considered to take place by copy choice (strand switching) (Kirkegaard & Baltimore, 1986), has been demonstrated between PVs of vaccine- and wild-type origin (Cammack *et al*., 1988; Furione *et al*., 1993). Recent phylogenetic analysis of complete enterovirus genomes provided evidence suggesting that recombination also occurs between other enteroviruses, and that intraspecies recombination is a relatively frequent

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event in the evolution of enterovirus genomes (Santti *et al.*, 1999).

The high mutation rate of enteroviruses enables detailed molecular epidemiological studies based on comparison of relatively short regions of the genome. A 150 nucleotide (nt) sequence at the junction of the capsid and nonstructural proteins (90 nt encoding the VP1 capsid protein and 60 nt encoding the 2A protease) has been extensively used in molecular epidemiological studies of PVs, resulting in a large sequence database (Rico-Hesse *et al.*, 1987; Zheng *et al.*, 1993; Huovilainen *et al.*, 1995; Kew *et al.*, 1995; Mulders *et al.*, 1995; Chezzi *et al.*, 1997; Morvan *et al.*, 1997; Fiore *et al.*, 1998). The term 'genotype', which is defined as a cluster of genetically related viruses with less than 15% nucleotide divergence across the 150 nt sequence, has been used for discrimination of genetic lineages of PVs, whereas less than 2% nucleotide divergence between strains has been considered to indicate direct epidemiological linkage (Rico-Hesse *et al.*, 1987). It has been demonstrated that within each PV serotype the independent genotypes usually show a distinct geographical distribution, but that occasionally long-distance importation of PV strains occurs. In addition to PVs, the molecular epidemiology of CAV24 variant and enterovirus 70, pathogens which have caused large outbreaks of acute haemorrhagic conjunctivitis, has been studied by partial sequencing (Ishiko *et al.*, 1992; Takeda *et al.*, 1994). Furthermore, regression analysis of genetic distances between isolates has enabled estimation of the time of emergence of these viruses. Partial genomic sequencing has also been applied to the molecular epidemiological analysis of CBV1 (Zoll *et al.*, 1994), CBV4 (Hughes *et al.*, 1993), CBV5 (Kopecka *et al.*, 1995) and enterovirus 71 (Brown *et al.*, 1999), as well as EV30 (Gjøen *et al.*, 1996) which is characteristically involved in epidemics of meningitis.

Analysis of the CAV9 prototype strain (Griggs) revealed that, despite its CAV-like pathogenicity in newborn mice, it is genetically more closely related to CBVs than to other CAVs (Chang *et al.*, 1989; Pulli *et al.*, 1995). However, when compared to CBVs, the CAV9 genome has an apparent insertion of approximately 15 amino acids at the C terminus of the VP1 capsid protein. This insertion contains an RGD (arginine-glycine-aspartic acid) tripeptide, which is known to be the cell attachment motif of a number of adhesive extracellular matrix, blood and cell surface proteins, and is recognized by several integrins (Ruoslahti & Pierschbacher, 1987; Ruoslahti, 1996). In further studies it was demonstrated that the RGD motif, which was found to be fully conserved among five clinical CAV9 strains isolated in the UK over a period of 25 years (Chang *et al.*, 1992), mediates CAV9 attachment to $\alpha_v\beta_3$ integrin (Roivainen *et al.*, 1991, 1994). However, studies on trypsin-treated virus and virus mutants, which lack the RGD motif, indicated that the virus is able to use an RGD-independent pathway in cell entry (Roivainen *et al.*, 1991; Hughes *et al.*, 1995). The C-terminal region of the VP1 capsid protein was also shown to be antigenic by using a peptide-scanning technique, but it was found that the RGD motif itself was poorly immunogenic whereas antibody-binding sites were located at both sites of the motif (Pulli *et al.*, 1998*a*, *b*).

In this study, 35 clinical isolates of CAV9, collected from different geographical regions during the last five decades, were subjected to molecular analysis. To explore phylogenetic relationships between the isolates in various genomic regions and to analyse variation, in particular at the C terminus of the VP1 protein, three regions, one covering the hypervariable part of the 5′NCR, VP4 gene and part of the VP2 capsid protein-coding region, another covering the junction region of the VP1 capsid protein and 2A protease genes, and the third encoding part of the 3D polymerase, were selected for RT–PCR amplification and direct sequencing.

Methods

Viruses. Thirty-five clinical isolates of CAV9 (Table 1) were obtained from the specimen collections of the Departments of Virology at the Universities of Turku and Helsinki (Finland), from the Centers for Disease Control and Prevention (CDC, Atlanta, USA; provided by Steven Oberste and Mark Pallansch), and from the National Institute of Public Health and the Environment (RIVM, The Netherlands; provided by Harrie van der Avoort). Specimens obtained from CDC and RIVM were passaged once in RD (human rhabdomyosarcoma) cells before further analysis. Passage history of the Finnish isolates included one to four rounds in various cell lines. Prior to molecular analysis, all 35 isolates were typed by a microneutralization test using CAV9 type-specific antiserum obtained from the ATCC.

RNA extraction, RT–PCR and sequencing. RNA was isolated from 100 µl of specimen using the Ultraspec method (Cinna/Biotecx Laboratories Inc.) with a slightly modified protocol of the manufacturer's instructions (Santti *et al.*, 1997). cDNA was generated in a 40 µl reaction volume for 1 h at 37 °C using the specific antisense primer (see below) and M-MLV RT (Promega) according to the instructions provided. Ten µl from the cDNA reaction was added to the PCR reaction mixture, which had a total volume of 100 µl and contained 10 mM Tris–HCl (pH 8·8), 50 mM KCl, 1·5 mM $MgCl_2$, 0·1% Triton X-100, 0·2 mM dNTPs, 50 pmol upstream and downstream primers and 1 U of Dynazyme thermostable DNA polymerase (Finnzymes). The reactions were run in a Perkin-Elmer Cetus thermal cycler. The cycle profile included 40 cycles of 2 min denaturation at 94 °C, 2 min annealing at 55 °C and 4 min elongation at 72 °C.

A 652 bp amplicon representing the 5′NCR, VP4 and VP2 capsid protein-coding regions (nt 547–1198; numbering according to the CAV9 Griggs strain; Chang *et al.*, 1989) was obtained using primers 4+ (sense; 5′ TACTTTGGGTGTCCGTGTTTC) and 5−n (antisense; 5′ GGBAAYTTCCACCACCANCC). The VP1/2A junction region of the genomes was amplified using primers VP1+ (sense; 5′ ACCAGAGCTTGGGTGCCGCG) and 2A− (antisense; 5′ ACAACACCTTCNCCNCCCAT), which produce a 560 bp amplicon (nt 3180–3739). Two specimens (Net/1/64 and Net/1/79) that did not react with these primers were amplified using primers VP1+n (sense; 5′ AACCCCAGCRTNTTYTGGAC) and 2A−n (antisense; 5′ GTCTCTRTTRTAATCYTCCCA), which give rise to a 516 bp PCR product (nt 2946–3461). Primers 3D+ (sense; 5′ TTTGAYTACWCWGGNTATGATGC) and 3D− (antisense; 5′ WGSRTTCTTKGTCCATC) were used to amplify nt 6654–7184 (531 bp), which encode part of the 3D polymerase. The amplicons were purified using the Qiaex II Gel Extraction Kit (Qiagen) and sequenced with fluorescent dye-labelled terminators using an

Table 1. CAV9 isolates used in the analysis

Strain	Origin	Year of collection	Specimen*	Clinical data†
Can/1/85	Canada	1985	NK‡	Meningitis
Can/1/86	Canada	1986	NK	Headache, diarrhoea
Den/1/71	Denmark	1971	NK	NK
Fin/1/83	Finland	1983	F	–
Fin/2/83	Finland	1983	L	–
Fin/1/88	Finland	1988	MEF	Fever, respiratory symptoms
Fin/1/93	Finland	1993	PS	Fever, tonsillitis, gastric pain
Fin/2/93	Finland	1993	F	–
Fin/1/94	Finland	1994	F	Cerebellitis
Fin/2/94	Finland	1994	L	Fever
Fin/1/95	Finland	1995	PS	Fever, headache
Fin/2/95	Finland	1995	L	Fever, vomiting
Fin/1/96	Finland	1996	F	Fever, exanthema
Fin/2/96	Finland	1996	L	Encephalitis
Fin/3/96	Finland	1996	F	Meningitis
Fin/1/97	Finland	1997	L	–
Fin/2/97	Finland	1997	F	Meningitis
Hon/1/77	Honduras	1977	NK	NK
Mex/1/75	Mexico	1975	NK	NK
Net/1/59	Netherlands	1959	F	Fever, exanthema
Net/1/61	Netherlands	1961	F	Fever, neck stiffness
Net/1/63	Netherlands	1963	PS	Fever, convulsions
Net/1/64	Netherlands	1964	F	Gastroenteritis, pharyngitis
Net/1/67	Netherlands	1967	F	Myelitis
Net/1/71	Netherlands	1971	PS	Meningitis
Net/1/73	Netherlands	1973	F	Meningitis
Net/1/78	Netherlands	1978	PS	Meningitis
Net/1/79	Netherlands	1979	PS	Meningitis
Net/2/79	Netherlands	1979	F	Pneumonia
Net/1/81	Netherlands	1981	F	Respiratory infection
Thai/1/93	Finland/Thailand§	1993	F	–
US-CO/1/74	Colorado, USA	1974	NK	NK
US-MD/1/88	Maryland, USA	1988	NK	NK
US-MS/1/78	Mississippi, USA	1978	NK	NK
US-NC/1/83	North Carolina, USA	1983	NK	NK

* F, stool specimen, L, cerebrospinal fluid, MEF, middle ear fluid, PS, pharyngeal specimen.
† –, no symptoms.
‡ NK, not known.
§ Child adopted from Thailand; specimen collected 2 days after arrival at Finland.

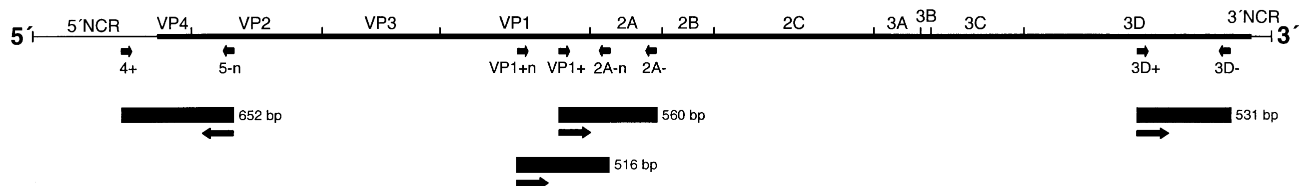

Fig. 1. Schematic representation of the amplification and sequencing strategy used for the studied CAV9 isolates. Genome organization is shown on the top (according to Chang *et al.*, 1989). Attachment sites and codes of the oligonucleotide primers are shown below the genome as short arrows; black rectangles indicate the resulting amplicons. The numbers refer to the sizes of the amplicons. Primers used for sequencing are represented as longer arrows. In the amplification of the VP1/2A junction region, PCR products of the expected size were obtained from 33 out of the 35 isolates studied using primers VP1+ and 2A−. The two strains (Net/1/64 and Net/1/79) which failed to react with these primers were successfully amplified using primers VP1+n and 2A−n.

automated DNA Sequencer (Applied Biosystems) according to the manufacturer's instructions. Primers 5 − n, VP1 +, VP1 + n and 3D + were used in the sequencing reactions. A schematic representation of the amplification and sequencing strategy is shown in Fig. 1.

Analysis of the sequence data. Pairwise nucleotide identities were calculated using the program Gap in the GCG software package (Devereux *et al.*, 1984). Multiple sequence alignments as well as dendrograms for the 5′NCR and the 150 nt sequence at the junction region of the VP1 and 2A genes were generated using the PileUp program (GCG). Bootstrapped phylogenetic trees of 1000 replicates were constructed from the alignments by the neighbour-joining method of Saitou & Nei (1987) as implemented in the Clustal X program (Thompson *et al.*, 1997). Sequences reported in this paper have been deposited in GenBank under the following accession numbers: AF166172–AF166253 and AF224645–AF224661.

Results

RT–PCR and sequencing

A 500 nt long sequence covering the hypervariable part of the 5′NCR, the VP4 gene and part of the VP2 capsid protein-coding region (nt 655–1154 in CAV9 Griggs strain) was determined for all 35 CAV9 isolates (Fig. 1). Sequence of approximately 390 nt, representing the junction region of the VP1 capsid protein and the 2A protease genes (nt 3228–3618), was obtained from 33 of the 35 isolates. The two strains (Net/1/64 and Net/1/79) which failed to react with the first combination of primers (VP1 + /2A −) were successfully amplified using alternative primers (VP1 + n/2A − n), which resulted in a sequence of 213 nt (nt 3228–3440) that overlapped with those of the other isolates studied. A 441 nt sequence encoding part of the 3D polymerase (nt 6720–7160) was also determined from 29 isolates.

Genotypes found in the analysis of the VP1/2A junction region

PV genotypes have been defined as clusters of related strains with < 15% nucleotide divergence in a 150 nt long sequence at the junction region between the VP1 capsid protein and the 2A protease genes (Rico-Hesse *et al.*, 1987). By comparing an analogous region of the CAV9 genome (nt 3258–3407), the studied isolates and the Griggs strain could be assigned to 12 genotypes, which were designated I–XII (Roman numerals) in the chronological order of the isolation of the earliest virus strain in each genotype (Fig. 2). The maximum nucleotide divergence between the isolates was 34%, which was seen between strains Net/1/67 and US-MD/1/88. The prototype Griggs strain, which was isolated in the early 1950s, was the only representative of genotype I. Genotype II included the earliest isolates from the Netherlands from 1959–1967, which share a minimum of 89% nucleotide identity, and, in addition, an isolate from Denmark from 1971, which exhibits on average 88% identity with the Dutch isolates. Strains Net/1/59 and Net/1/61 share 98% identity in this genomic region, which is indicative of a direct

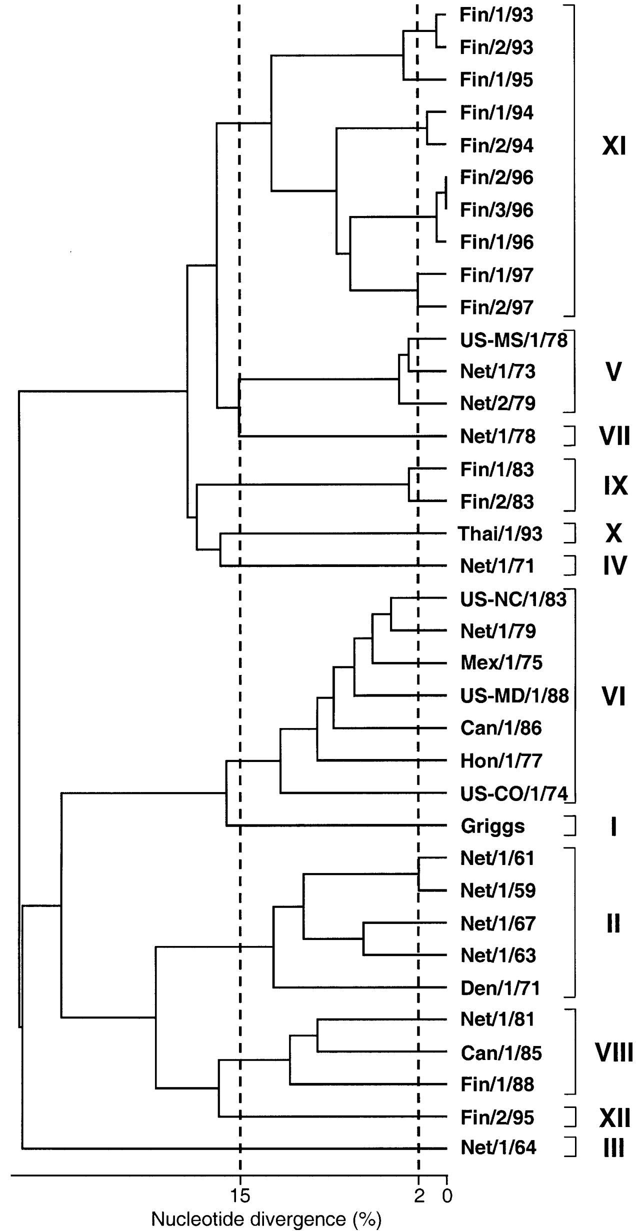

Fig. 2. Dendrogram illustrating sequence relationships among the studied CAV9 isolates and the prototype Griggs strain (Chang *et al.*, 1989) based on analysis of a 150 nt fragment at the VP1/2A junction region (nt 3258–3407; Griggs strain). Genotypes, defined as clusters of related sequences with less than 15% nucleotide divergence, are grouped within brackets and assigned roman numerals I–XII. Less than 2% nucleotide divergence is considered to indicate direct epidemiological linkage.

epidemiological linkage between the isolates. Genotype III was represented by one isolate (Net/1/64) which is distant from all the other strains analysed. An isolate from the Netherlands from 1971 forms genotype IV. It shows 84% nucleotide identity to a geographically and temporally distant isolate from Thailand from 1993 (genotype X).

Genotypes V, VI and VIII are geographically the most widespread. Genotype V contains three closely related strains;

one from the United States from 1978 and two from the Netherlands from 1973 and 1979, which share a minimum of 97% nucleotide identity. Genotype VI comprises isolates from the United States (1974–1988), Mexico (1975), Honduras (1977), the Netherlands (1979) and Canada (1986). The isolate from the Netherlands and an isolate from North Carolina, USA, from 1983 are the most closely related of these strains sharing 96% nucleotide identity. An isolate from The Netherlands from 1978 represents genotype VII. It shows on average 85% nucleotide identity with genotype V isolates, and could, in principle, also be assigned to this genotype. Genotype VIII contains one isolate from the Netherlands from 1981, one from Canada from 1985 and one from Finland from 1988 which share 87–91% nucleotide identity. Genotype IX contains two strains from Finland from 1983 which show approximately 3% nucleotide divergence (4 mutations/147 positions). These strains were isolated from two patients in a 3 day period.

The largest number of virus strains included in the study originated from Finland. Ten of the isolates, from 1993–1997, represent genotype XI. Strains isolated within each year share a minimum identity of 98% which, again, indicates direct epidemiological linkage. The only isolate from 1995 in this genotype is most closely related to the two isolates from 1993 sharing on average 97% nucleotide identity with these strains. Another isolate from Finland from 1995 represents genotype XII, which is distant from genotype XI but closer to the genotype VIII strains.

Phylogenetic analysis of the isolates

Phylogenetic grouping in the VP4/VP2 region. To investigate the evolutionary relationships between the CAV9 isolates in the VP4/VP2-coding region, a neighbour-joining tree based on analysis of nt 744–1154 (Griggs strain) of the CAV9 strains together with 11 representatives of other cluster B enteroviruses was constructed (Fig. 3). The two strains of CBV3 and CBV4 as well as EV9 clustered together with high bootstrap values. A group consisting of all the CAV9 isolates was supported by a bootstrap value of 61% whereas a subgroup containing all the other CAV9 isolates except the Net/1/64 strain was slightly better supported (74%). In general, virus strains within the CAV9 group segregated similarly to that observed in the analysis of the 150 nt interval at the VP1/2A junction region (Fig. 2). Virus groups representing genotypes V, VI, IX and XI were supported by high bootstrap values. However, in the case of genotype II, only the relationship between the Dutch isolates was highly supported (100%; data not shown) whereas the whole group was supported in 63% of the bootstrap replicates. The bootstrap value for the group of strains representing genotype VIII was low (42%; data not shown); only clustering of strains Fin/1/88 and Net/1/81 was significantly supported. Of the remaining genotypes which were all represented by a single isolate, a monophyletic group consisting of genotype IV and X isolates was highly supported. In addition, statistically significant clustering was seen between genotypes I and VI. The maximum nucleotide divergence between the CAV9 isolates was 22%, which was observed between Net/1/64 and Can/1/85 as well as Net/1/67 and Fin/2/93.

VP1/2A region. A neighbour-joining tree was constructed from the nucleotide sequences encoding the VP1/2A junction region (nt 3228–3618 in the Griggs strain) of the CAV9 strains and other cluster B viruses (Fig. 3). The bootstrap analysis revealed that all the CAV9 strains belong to a monophyletic group. The relationship between the two CBV3 strains was also statistically significant whereas the bootstrap values for the groups containing the two CBV4 as well as the two EV9 strains were relatively low (54 and 47%, respectively; data not shown). Analysis of the CAV9 strains revealed a grouping pattern similar to that obtained in the analysis of the 150 nt sequence from the same region (Fig. 2) as well as that obtained in the analysis of the VP4/VP2 region (Fig. 3). Virus genotypes V, IX and XI were supported by high bootstrap values. In contrast to the results obtained in the analysis of the VP4/VP2 region, the bootstrap value for the virus strains constituting genotype VI was relatively low (59%; data not shown) whereas a larger group containing genotype I and VI viruses was highly supported. Also differently from the VP4/VP2 region, viruses representing genotype VIII formed a monophyletic group that was highly supported by the bootstrap analysis. Furthermore, the relationship between the genotype II strains was also highly supported whereas the relationship between genotype IV and X viruses was not statistically significant (38%; data not shown). Viruses constituting genotypes VIII and XII clustered together showing a high bootstrap value which was consistent with the results obtained in the analysis of the shorter (150 nt) fragment. The maximum nucleotide divergence between the CAV9 isolates in this genomic region was 27%, which was seen between strains Net/1/67 and US-CO/1/74.

3D region. To examine the evolutionary relationships of the CAV9 isolates at the 3′-terminal part of the genome, sequence encoding part of the 3D polymerase (nt 6720–7160 in Griggs strain) was determined from 29 isolates which represented 10 of the 12 genotypes recognized in the analysis of the VP1/2A region. Phylogenetic analysis of the CAV9 strains together with other representatives of cluster B viruses revealed a strikingly different grouping pattern compared to that obtained in the analysis of the other coding regions (Fig. 3). The CAV9 isolates did not cluster together, but they were instead interspersed among the other strains studied. In addition, none of the five genotypes, which were represented by more than one strain, formed monophyletic groups. In contrast, some groups, which contained both CAV9 isolates as well as other members of the enterovirus genus, were highly supported. The analysis also detected the close relationship between the

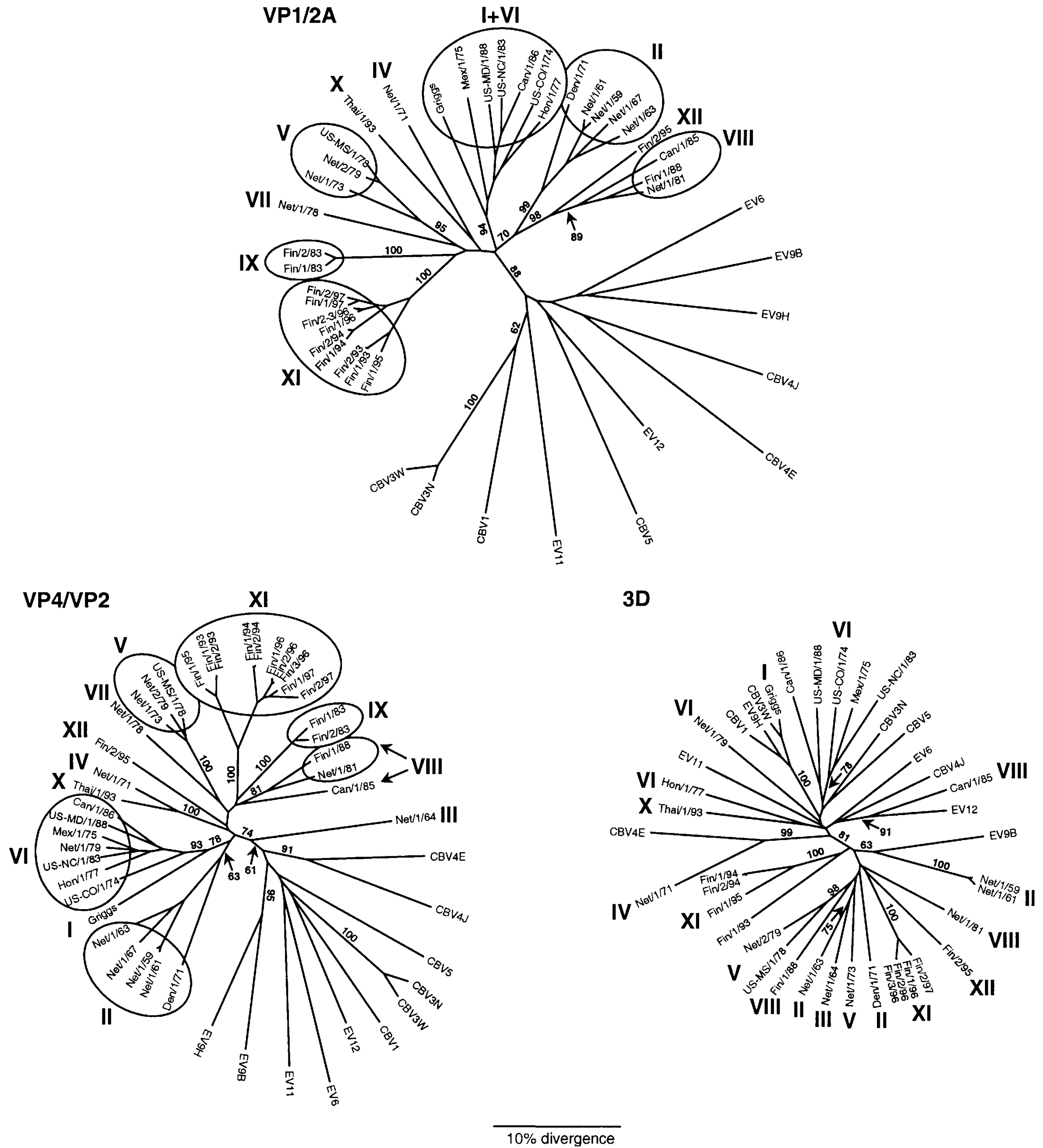

Fig. 3. Neighbour-joining trees constructed from nucleotide sequences encoding the junction region between the VP1 capsid protein and 2A protease (nt 3228–3618; numbering according to CAV9 Griggs strain; Chang *et al*., 1989), the VP4 polypeptide and part of the VP2 capsid protein (nt 744–1154) and part of the 3D polymerase (nt 6720–7160) of the studied CAV9 isolates. The prototype CAV9 Griggs strain together with selected representatives of other cluster B enteroviruses were included in the analysis. Strains Net/1/64 and Net/1/79, for which only the sequence of the first 213 nt from the 5′ end of the VP1/2A region was determined, were excluded from the analysis of this genomic region. No amplicon was obtained from strains Net/1/67, Net/1/78, Fin/1/83, Fin/2/83, Fin/2/93 and Fin/1/97 in the analysis of the 3D region. Bootstrap probabilities for the major clusters are indicated (only values exceeding 60% of the replicates are shown). Genotypes I–XII, defined by the analysis of a 150 nt sequence covering the VP1/2A junction region (Fig. 2), are indicated. The scale for the genetic distances is indicated. Previously published full-length sequences of CAV9 Griggs strain (D00627), CBV1 (M16560), CBV3 Nancy (CBV3N; M33854) and Woodruff (CBV3W; U57056) strains, CBV4 E2 (CBV4E; S76772) and JVB (CBV4J; X05690) strains, CBV5 (X67706), EV6 (U16283), EV9 Barty (EV9B; X92886) and Hill (EV9H; X84981) strains, EV11 (X80059) and EV12 (X79047) were used in the comparisons.

Griggs prototype strain, CBV1, CBV3W and EV9H, that has been reported previously (Santti *et al*., 1999). The maximum nucleotide divergence between the CAV9 isolates in this genome region was 23% which was seen between strains Net/1/71 and US-MS/1/78.

Amino acid sequence diversity among the isolates

The deduced amino acid sequences of the VP4/VP2, VP1/2A and 3D regions of the studied CAV9 sequences were aligned with the prototype Griggs strain together with eight representatives of other cluster B viruses as well as three antigenic variants (VP1/2A alignment is shown in Fig. 4). The VP4/VP2 amino acid sequences were highly conserved among these virus strains. Most of the amino acid differences in the VP4 capsid protein, which in picornaviruses is positioned internally in the virion, were concentrated on a relatively short region of the polypeptide between codons 16–24. Variation in the N terminus of the VP2 capsid protein was limited to 19 of the 68 sites.

Analysis of the VP1 capsid protein sequences revealed that, when compared to CBV3N, all the studied CAV9 isolates had an extension of 14 or 15 amino acids to the C terminus of the polypeptide (Fig. 4). The RGD (arginine-glycine-aspartic acid) tripeptide was fully conserved among the isolates whereas residues surrounding the motif were highly variable. Only glutamine at position −4 (RGD is positions 1–3) and threonine at position +9 were invariant. Moreover, serine at position −3 was almost invariant; only strain Net/1/64 had an alanine substitution at this site. Genotype VI isolates had a leucine to phenylalanine substitution at position +7. Different substitutions were most frequently seen at positions −5, −2, +5 and +6. Four differences were seen at position −5 and, in addition, 18 of the isolates had an apparent deletion at this site. Six different amino acids were seen at position −2, five at position +5 and eight at position +6. The RGD tripeptide was preceded by either a methionine or a leucine. Residues preceding the extension were more conserved among the CAV9 isolates whereas other members of cluster B enteroviruses differed clearly from the CAV9 strains in this region (Fig. 4).

Substitutions in the 2A region were relatively evenly distributed (Fig. 4). Histidine at position 21 and aspartate at position 39, which together with a cysteine residue form the catalytic triad of the entero- and rhinovirus 2A proteases (Bazan & Fletterick, 1988; Yu & Lloyd, 1991), were fully conserved. Little variation was seen in the 3D polymerase (data not shown). The YGDD sequence, which has been hypothesized to be at or near the catalytic site of the molecule (Kamer & Argos, 1984; Jablonski *et al*., 1991), was fully conserved among the isolates.

Analysis of the hypervariable region

The hypervariable region, covering approximately 100 nt preceding the polyprotein initiation codon, is the most variable part of the enterovirus genome (Toyoda *et al*., 1984; Rivera *et al*., 1988). For example, the nucleotide sequences of the three PV Sabin strains differ from each other by about 50% in this region (Toyoda *et al*., 1984). It has been suggested that this segment serves as a spacer region between the internal ribosome entry site and the polyprotein initiation codon; the ribosome scans through this region for the initiation site. The lack of stable secondary structures and initiation codons in this region are consistent with this hypothesis (Rivera *et al*., 1988).

A dendrogram illustrating the relationships of the CAV9 strains in the hypervariable region (corresponds to nt 655–743 of the Griggs strain) is shown in Fig. 5. The virus grouping was highly similar to that observed in the analysis of the 150 nt sequence at the VP1/2A junction region. The maximum nucleotide divergence between the isolates was 51%, which was seen between strains Net/1/67 and US-NC/1/83 as well as Net/1/67 and Hon/1/77. Despite the high overall diversity, strains within each VP1/2A region genotype shared > 85% nucleotide identity. The strain Den/1/71 was an exception as it showed only 58% nucleotide identity to other genotype II isolates. In addition, strain Net/1/78 was slightly more distant from genotype V strains in this region (75% identity) than in the VP1/2A region (85% identity) whereas genotype VIII and XII viruses shared 85% nucleotide identity in this region.

Discussion

Molecular techniques have been extensively used to study the epidemiology of polioviruses and the results obtained have had a significant impact on the worldwide efforts to eradicate poliomyelitis (for review, see e.g. Kew *et al*., 1995). Much less is known about the molecular epidemiology of other enteroviruses. The aim of the present study was to characterize the epidemiology and evolution of coxsackievirus A9, which is one of the most frequently isolated enterovirus serotypes in clinical diseases (Strikas *et al*., 1986; Hovi *et al*., 1996). CAV9 strains isolated during the last five decades from different parts of the world were studied by partial genomic sequencing.

Phylogenetic analysis of the CAV9 isolates together with 11 representatives of other cluster B enteroviruses revealed that the CAV9 strains formed a monophyletic group in the VP4/VP2 and VP1/2A regions but not in the 3D region. In the VP1/2A region, the CAV9 group was supported by a bootstrap value of 88% while in the VP4/VP2 region the group was found in 61% of the 1000 bootstrap replicates. These findings are consistent with previous observations that the serotype specificity is reflected in the capsid protein-coding sequence but not necessarily in other parts of the genome (Zoll *et al*., 1994; Kopecka *et al*., 1995; Santti *et al*., 1999). Furthermore, these results correlate well with the findings of Oberste *et al*. (1998, 1999), which indicate that the VP1 sequence represents the serotype identity better than the sequence of the VP4/VP2 junction region does.

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                         261            VP1                   299 1                                 2A                                                    91
I     Griggs      AFSVDFTPTPITDTRKDINTVT-TVAQSRRRGDMSTLNTHGAFGQQSGAVYVGNYRVINRHLATHTDWQNCVWEDYNRDLLVSTTTAHGCDVIARCQCTTGVYFCASKNKHYPVSFEGPGLVEVQESEYYP
II    Net/1/59    ......................-.IV..EH...L.A......Y.H...........I............Q...........I.........T...............R.......................
      Net/1/61    ....................I.-.IV..EH...L.A......Y.H...........I............Q...........I.........T...............R.......................
      Net/1/63    ......I...............-.I...E....L.D.S....Y.H............V...........Q...........I.........T...............RG......................
      Net/1/67    ......................-.IV..E....L.D.S....Y.H............V.......A...R...........I.........T.......A...Y...R............I..........
      Den/1/71    ......................-.IV..E......A......Y.H........................Q...........I.........T....K..A...Y...R.......................
III   Net/1/64    ......................-NIT.ATY...LHA.S..................IV.......N...S..-----------------------------------------------------------
IV    Net/1/71    ......................-SI-..K......I.S..................LV.......A.........................T....H.N...............A................
V     Net/1/73    ......................-SI-..G......E..............I......LV.......V.........................T.............T.R......T................
      US-MS/1/78  ......................-SI-..G......E..............I......LV.......V.........................I.............Q.R............I..........
      Net/2/79    ......................-SI-..G.....FE..............I......LV.......V.........................T.............H.R............I..........
VI    US-CO/1/74  ...............E...A..-S............F.............I.......V.......A.......D.................I.......................................
      Mex/1/75    ......................-S....G.......F.............I.......V.......A.........................T.............S.........................
      Hon/1/77    ...................A..-S.T..G.......F.............I.......V.......A.........................I....R..................................
      Net/1/79    ...................S.S-S.T..G.......F.............I.......V.......A......-----------------------------------------------------------
      US-NC/1/83  ...................S..-S.T..G.......F.............I.......V.......A.........................I...............R.......................
      Can/1/86    ................H..S..-S.T..........F.............I.......V......RA..................I......................R.......................
      US-MD/1/88  ...................S..-S.T..G....L..F.............I.......V.......A.........................I...............R.......................
VII   Net/1/78    ......................-SI-..EH.....G..............A......LV.......A.........................T......S........R.......................
VIII  Net/1/81    ......................-.IV..G....LAA........H....I.....K.V.......A........E......I.........T......................T................
      Can/1/85    ......E...............-.IV..G....LTA...Y....H....I.....K.V.......A........E......IC........T......................N................
      Fin/1/88    ......................-.IV..EH...LAA........H....I.....K.V.......A........E......I.........T......................T................
IX    Fin/1/83    ......................-S.-..GL.....V.....................V.......C.....I...................T................S......................
      Fin/2/83    ......................-SI-..EL.....V.....................V.......C.....I...................T.......................................
X     Thai/1/93   ......A...............-SI-..EH.....A.H...V...............V.......K.........................T......SA.......R......X................
XI    Fin/1/93    ...............I......-SI-..G......A.....................V.......D.........................T...............RS..F...................
      Fin/2/93    ...............I......-SI-..G......A.....................V.......D.........................T...............RS..F...................
      Fin/1/95    ...I..................-SI-..G......A.....................V.......D.........................T...............R...F...................
      Fin/1/94    ......................-SI-..V.....TA.....................V.......N...S.....C...............T...............R.............L.........
      Fin/2/94    ......................-SI-..V....LTA.....................V.......N...G.....................T...............R.......................
      Fin/1/96    ......................-SI-..G......A.....................V.......N...S.....................T...............R.......................
      Fin/2/96    ......................-SI-..G......A.....................V.......N...S.....................T...............R.................D.....
      Fin/3/96    ......................-SI-..G......A.....................V.......N...S.....................T...............R.......................
      Fin/1/97    ......................-SI-..G......M.....................V.......N...S.....................T...............R.R.....................
      Fin/2/97    ......................-SI-..G......M.....V...............V.......D...S.....................T...............R.R.....................
XII   Fin/2/95    ......................-.IE..EH...LAA........H....I......IV......R.........E.......C........T.......A..............Y................
      CBV1        QKN.N.N..GV.T..SN.T-T------------------T.................V......RE...R.....................I...............R.......................
      CBV3N       .KN.N.Q.SGV.T..QS.T-T---------------MTNT.................V......SA........S................I.....................I.................
      CBV3W       .KN.N.Q.SGV.T..QS.T-T---------------MTNT.................V......SA........N................I....R................I......I..........
      CBV4E       .KN.N.DVEAV.TE.ASLV-T------------------T.PH..............V.......F....YI...................T.......S.....V.R......V................
      CBV4J       .K..N.DVEAV.AE.ASLI-T------------------T.PY.H..........K.V.......V.........................T................S......................
      CBV5        .GN.N.E..GV.ES.TE.T-A---------------MQ.T.VL...T..ICI.....V......SE...R.....................T....R.S........R.......................
      EV6         KDN..YE.KGV.TS.TS.T-I.NSKH----------ME.........A.......V.................................T....H..........R...H..V............G...
      EV9B        KAN.N.EA.AF.E..DT....PVSNHG.G....LAA.S.......E...........L.............I...................T......K...F..Q.........................
      EV9H        KAN.N.EA.AV....DT....PLSTHGVS.------------..Y.H....R......IV...........K.....................T.................................A.....
      EV11        .ST.N....NV..K.TS..YIPE..KPD--------.SNY....Y.......V....V.......................I..............R.S....Y.Q..G.....N................
      EV12        SSN.N.K..DV.TS.TS.T-EVPSLRP.--------VVNT.........A.......V.......V.........................T...............R.......................
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Fig. 4. Alignment of amino acid sequences of the junction region between the VP1 capsid protein and 2A protease of the studied CAV9 isolates. Corresponding regions of CAV9 Griggs strain and 11 other members of species B enteroviruses were also included in the alignment. Dots represent amino acid residues identical to those of the CAV9 Griggs sequence while dashes indicate gaps. Genotypes I–XII, which were defined by the analysis of a 150 nt fragment at the VP1/2A junction region (Fig. 2), are indicated on the left. The vertical bar represents the putative cleavage site between individual viral proteins. The highly conserved RGD tripeptide at the C-terminal part of the VP1 capsid protein as well as the conserved histidine and aspartate residues of the 2A protease found near or at the active site of the molecule are shaded.

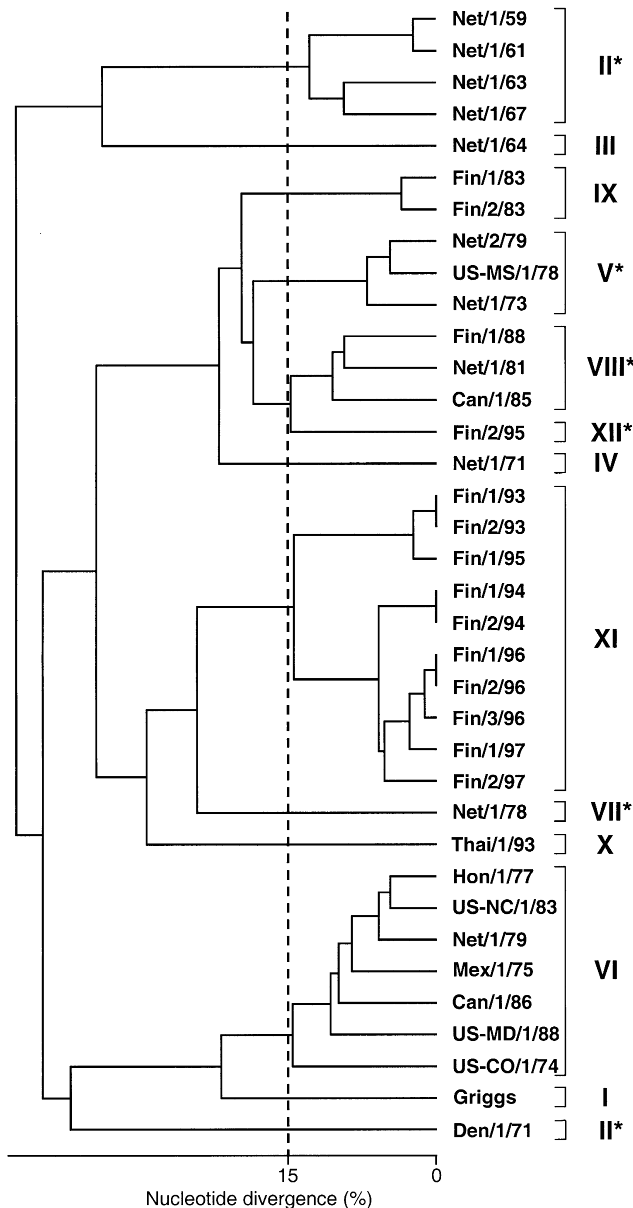

Fig. 5. Dendrogram showing genetic relationships among the studied CAV9 isolates and the prototype Griggs strain based on analysis of the hypervariable part of the 5′NCR (nt 655–743; Griggs strain). Genotypes I–XII, defined as clusters of strains sharing > 85% nucleotide identity across a 150 nt fragment at the VP1/2A junction region (Fig. 2), are indicated. Genotypes that show different grouping compared to the analysis of the VP1/2A region are indicated by asterisks.

The CAV9 isolates could be divided into 12 genotypes using the criteria designated for PVs (Rico-Hesse *et al.*, 1987) that strains sharing > 85% identity in a 150 nt sequence at the VP1/2A junction region belong to the same genotype (Table 2). While most of the strains within each genotype showed geographical clustering, the analysis also showed evidence of long-distance importation of virus strains. In fact, three out of the six genotypes which were represented by more than one strain contained isolates both from Europe and the Americas. This finding suggests that individual CAV9 genotypes are frequently found over a wide geographical region, although more precise evaluation of geographical distribution of CAV9 genotypes cannot be made from this relatively limited data. For example, it cannot be concluded that genotype XI was geographically restricted to Finland since the study material did not contain any isolates from this time from any other country. It is also difficult to estimate the number of CAV9 genotypes which co-circulate at a given time. However, at least four genotypes were present in the Netherlands from 1978 to 1981. Appearance of several co-circulating genotypes is a typical phenomenon, at least for PVs in endemic countries with low vaccine coverage (Huovilainen *et al.*, 1995), but probably also occurs in other enteroviruses. Reliable estimation of the evolutionary rate of enterovirus genomes is usually complicated by the existence of multiple co-circulating lineages. In rare cases, when the founder virus has been identified, such calculations have, however, been possible. The VP1/2A sequence of PV type 1 introduced into the Andean region of South America in 1980 was estimated to have evolved at a nearly constant rate of 9×10^{-3} nt substitutions/site/year (Kew *et al.*, 1995). Accordingly, the maximum time for the existence of a given genotype has been evaluated to be approximately 17 years. Interestingly, all CAV9 genotypes found in the present study followed this rule.

Table 2. Geographical distribution of CAV9 genotypes

Genotype	Year	Country (no. of isolates)
I	Early 1950s	USA (1)
II	1959–1971	Denmark (1), Netherlands (4)
III	1964	Netherlands (1)
IV	1971	Netherlands (1)
V	1973–1979	Netherlands (2), USA (1)
VI	1974–1988	Canada (1), Honduras (1), Mexico (1), Netherlands (1), USA (3)
VII	1978	Netherlands (1)
VIII	1981–1988	Canada (1), Finland (1), Netherlands (1)
IX	1983	Finland (2)
X	1993	Thailand (1)
XI	1993–1997	Finland (10)
XII	1995	Finland (1)

Phylogenetic analysis of a longer region (approximately 390 nt) around the VP1/2A junction revealed that the designated genotypes actually represented phylogenetic lineages. The phylogenetic grouping pattern of the CAV9 strains observed in the analysis of the VP4/VP2 region was highly similar to that obtained in the analysis of the VP1/2A region. Differences, which could potentially be due to a recombinant origin of some of the virus strains, were mainly

observed in the phylogenetic relatedness of individual genotypes. For example, genotypes VIII and XII were monophyletic in the VP1/2A region but not in the VP4/VP2 region. Similarly, the relationship between genotypes IV and X was highly supported by bootstrap analysis in the VP4/VP2 (100%) region but not in the VP1/2A (38%) region. In the hypervariable region, which precedes the VP4 gene, genotypes VIII and XII clustered together whereas genotypes IV and X did not, which suggests that, if the observed differences were due to recombination, one of the recombination breakpoints could in both cases have been situated near or at the initiation codon. Recombination could also explain the relationship between strain Den/1/71 and other members of genotype II, which in the VP1/2A region form a monophyletic group with a bootstrap value of 99% but in VP4/VP2 the value decreases to 63%, apparently due to the divergence of Den/1/71 from the other strains. Finally, in the hypervariable region Den/1/71 is clearly separated from the other strains of genotype II. The finding of several co-circulating CAV9 genotypes also provides indirect evidence for the possibilities of recombination events.

The different phylogenetic grouping of the CAV9 isolates in the 3D region compared to other genomic regions could most easily be explained by the occurrence of multiple recombination events between members of cluster B. This is supported by a recent analysis of 17 complete enterovirus genomes belonging to cluster B which detected several regions of higher than average sequence homology between individual virus strains, suggesting that intraspecies recombination occurs relatively frequently in the evolution of enterovirus genomes (Santti *et al*., 1999). This analysis also suggested that most of the putative recombination breakpoints between different serotypes were located at the region encoding the nonstructural proteins. It needs to be kept in mind, however, that the number of completely sequenced enterovirus genomes is still limited and restricted mainly to prototype strains isolated decades ago. More extensive analysis of different isolates may reveal currently undiscovered genetic relationships between and within the genetic clusters.

Previous studies have indicated that the RGD motif found within the C-terminal extension of the VP1 capsid protein of CAV9 (Chang *et al*., 1989) is functionally significant and plays a role in early virus–cell interactions, because RGD-containing oligopeptides block CAV9 infectivity (Roivainen *et al*., 1991). Moreover, it has been demonstrated that the RGD motif mediates CAV9 attachment to $\alpha_v\beta_3$ integrin in GMK cells (Roivainen *et al*., 1994). Sequence analysis of five temporally highly distinct CAV9 clinical isolates from the UK demonstrated that the motif was fully conserved (Chang *et al*., 1992). The analysis also identified similarity of the VP1 extension of CAV9 to part of the precursor of human transforming growth factor β1, suggesting that the virus might have acquired this additional sequence by heterologous recombination with cellular sequences.

Previous investigations have also shown that infectivity of CAV9 is not completely abolished by removal of the RGD motif by trypsin (Roivainen *et al*., 1991) or by mutagenesis (Hughes *et al*., 1995), which indicates that the virus is able to use an RGD-independent pathway in its entry into the host cell. In the present study, the RGD triplet was found to be fully conserved among 35 CAV9 clinical isolates which suggests that the motif, although not essential for virus viability in cell culture conditions, plays a vital role in replication of the virus in humans. The amino acid residues surrounding the RGD triplet were highly variable among the isolates reflecting a high degree of structural flexibility. As already noted by Chang *et al*. (1992), the situation resembles that seen in foot-and-mouth disease virus (FMDV), another integrin-binding picornavirus, in which the RGD motif is in a surface-accessible location and forms part of the major antigenic site of the virion (Acharya *et al*., 1989; Fox *et al*., 1989; Berinstein *et al*., 1995). The RGD motif of FMDV has been shown to interact directly with some antiviral neutralizing antibodies (Verdaguer *et al*., 1995) whereas analysis of CAV9 suggested that antibodies bind most strongly to amino acid residues surrounding the RGD motif while the motif itself is poorly immunogenic (Pulli *et al*., 1998*a*, *b*).

In conclusion, the present analysis of the CAV9 isolates provides an overview of the molecular epidemiology and evolution of CAV9. While most strains within each genotype were found to cluster geographically, the analysis also detected long-distance importation of virus strains. Analysis of sequences derived from three different genomic regions demonstrated that the capsid protein but not the nonstructural protein-coding region correlates with the serotype. In addition, the invariant nature of the RGD sequence provides further evidence for its importance in the clinical pathogenesis of CAV9 infection.

We thank Steven Oberste, Mark Pallansch and Harrie van der Avoort for providing clinical isolates and Alexander Plyusnin and Glyn Stanway for their comments during the preparation of the manuscript. The study was supported by grants from the Academy of Finland, the Turku University Foundation, the Finnish Cultural Foundation and the Finnish Foundation for Research on Viral Diseases.

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Received 13 July 1999; Accepted 25 January 2000