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A nucleotide sequence comparison of coxsackievirus B4 isolates from aquatic samples and clinical specimens

M. S. HUGHES[1]*, E. M. HOEY[2] AND P. V. COYLE[3]

[1] *Department of Microbiology & Immunobiology, Queen's University of Belfast, Royal Victoria Hospital, Belfast 12, N. Ireland*

[2] *School of Biology and Biochemistry, Queen's University of Belfast, Medical Biology Centre, Belfast 9, N. Ireland*

[3] *Regional Virus Laboratory, Royal Victoria Hospital, Belfast 12, N. Ireland*

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SUMMARY

Ten coxsackievirus B4 (CVB4) strains isolated from clinical and environmental sources in Northern Ireland in 1985–7, were compared at the nucleotide sequence level. Dideoxynucleotide sequencing of a polymerase chain reaction (PCR) amplified fragment, spanning the VP1/P2A genomic region, classified the isolates into two distinct groups or genotypes as defined by Rico-Hesse and colleagues for poliovirus type 1. Isolates within each group shared approximately 99% sequence identity at the nucleotide level whereas $\leqslant 86\%$ sequence identity was shared between groups. One isolate derived from a clinical specimen in 1987 was grouped with six CVB4 isolates recovered from the aquatic environment in 1986–7. The second group comprised CVB4 isolates from clinical specimens in 1985–6. Both groups were different at the nucleotide level from the prototype strain isolated in 1950. It was concluded that the method could be used to sub-type CVB4 isolates and would be of value in epidemiological studies of CVB4. Predicted amino acid sequences revealed non-conservation of the tyrosine residue at the VP1/P2A cleavage site but were of little value in distinguishing CVB4 variants.

INTRODUCTION

Group B coxsackieviruses comprise six different serotypes. These viruses are associated with a wide range of illnesses including aseptic meningitis, encephalitis, pleurodynia, myocarditis and pericarditis [1]. Group B coxsackieviruses have been isolated from faeces, rectal swabs, cerebrospinal fluid and nasal secretions [2] and have been found in sewage and in sewage-contaminated water. A study of the aquatic environment of Northern Ireland (NI) for the presence of human enteroviruses in relation to public health in 1986–90, revealed the presence of CVB serotypes 2–5 [3]. CVB4 was the most commonly isolated serotype and represented 39% of 534 enterovirus isolates identified and 51·5% of 406 CVB isolates.

* Author for correspondence and reprints: Dr M. S. Hughes, Bacteriology Department, Veterinary Sciences Division, Stoney Road, Belfast BT4 3SD, N. Ireland.

Identification of 52 CVB isolates from clinical specimens submitted to the Regional Virus Laboratory, Belfast, during the same period showed the presence of the same four CVB serotypes in the community [3] with CVB4 constituting 25% of the identified isolates.

Given that infection by any one serotype may result in such a diverse spectrum of clinical diseases, it is plausible that variants within each serotype exist that differ antigenically and biologically. Populations of most RNA viruses represent a heterologous mixture of related genomes or quasispecies [4]. The natural occurrence of antigenic variants has been suggested by monoclonal antibody studies [5]. Little is known about the number of variants which can co-exist in populations and whether they can produce different disease syndromes. However, diabetogenic and non-diabetogenic variants of CVB4 have been described [6, 7] and myocarditic and non-myocarditic variants of CVB4 have also been reported [8].

It was not known whether, within each serotype, the CVB isolates derived from clinical and environmental sources in NI represented different strains. Rico-Hesse and colleagues [9] had been able to identify variants of wild type poliovirus 1 by nucleotide sequence analysis of a 150 nucleotide region spanning the VP1/P2A junction of the genome. Similar to polioviruses, the genome of CVB4 is composed of single stranded RNA which is translated into a polyprotein that is subsequently cleaved by viral proteases. Complete nucleotide sequences have been determined for CVB1 [10], CVB3 [11] and CVB4 [12]. Here a comparable study was attempted with CVB4 viruses, the most commonly isolated CVB virus from the aquatic environment in 1986–90, to determine whether the typing system utilized by Rico-Hesse and colleagues would be of value in differentiating strains of CVB4.

MATERIALS AND METHODS

Virus isolates

Six CVB4 isolates (1–6) recovered from the aquatic environment in NI in 1986–7, were selected for study. The samples were spatially or temporally distinct. Four CVB4 isolates (A–D) from clinical specimens submitted to the Regional Virus Laboratory, Belfast, were also included (Table 1).

Virus propagation and preparation of RNA

Virus isolates were plaque purified three times in Buffalo Green Monkey Kidney (BGM) cells [13]. For RNA preparation, confluent BGM monolayers, in 75 cm^2 flasks, were infected at a moi of 2·5 pfu/cell with plaque purified virus. Infected monolayers were incubated at 37 °C for 1 h with intermittent rocking. Eagles Minimum Essential medium (Gibco Ltd., Paisley, Scotland), containing 2% v/v foetal bovine serum, was added and the flasks reincubated at 37 °C until full cpe was apparent. Extraction of viral RNA was carried out according to the method of Rico-Hesse and colleagues [9] with the following modification. When full cpe was apparent the infected cells and tissue culture medium were harvested. The cells were separated from the medium by centrifugation at 1000 ***g*** for 5 min at 4 °C, subjected to two cycles of freeze–thawing and then resuspended in the medium.

Table 1. *Origin of* CVB4 *isolates*

Isolate code	Sample/specimen date	Sample/patient location	Patient		
			Age	Sex	Condition
1	29 Jan. 87	Craigavon Co. Armagh			
2	28 May 87	Portrush Co. Antrim			
3	20 Nov. 86	Kinnego Bay Co. Armagh			
4	6 Nov. 86	Craigavon Co. Armagh			
5	2 Apr. 87	Kinnego Bay Co. Armagh			
6	1 Sep. 86	Groomsport Co. Down			
A	18 Jul. 85	Bessbrook Co. Armagh	2 yr	M	Diarrhoea
B	21 Oct. 86	Richhill Co. Armagh	3 yr	M	Chronic diarrhoea
C	6 Jun. 85	Newry Co. Down	3 yr	M	Gastroenteritis
D	7 May 87	Belfast Co. Antrim	2 yr	F	Progressive lower limb paralysis; malignant glioma

Synthetic primers

Synthetic primers were prepared in an automated DNA synthesizer and purified by gel electrophoresis. Primer 1, 5′-CTCACTAGAAGGTCTCTATT-3′, was derived from the sequence (residues 3399–3418) beginning at 101 nucleotides to the 3′-side of the VP1/P2A junction of the prototype CVB4 strain [12]. Primer 2, 5′-GGACATACTTCCCAGG-3′, was derived from the sequence (residues 2556–2571) beginning 742 nucleotides to the 5′-side of the VP1/P2A junction. Primer 3, 5′-ATGTACGTGCCACCTGG-3′, was derived from the sequence (residues 2880–2896) beginning 418 nucleotides to the 5′-side of the VP1/P2A junction.

cDNA synthesis

The RNA pellet was resuspended in 10 μl of 10 mM methylmercury (II) hydroxide and 20 units of human placental ribonuclease inhibitor (Amersham International plc, England) and left to stand at room temperature for 15 min. Four μl of 700 mM 2-mercaptoethanol and 20 units of ribonuclease inhibitor were added and the reaction mix left for a further 5 min at room temperature. Ten μl of primer 1 (100 ng/μl) was hybridized to the RNA at room temperature for 5 min before the addition of 10 μl of a dNTP mix (20 μl of 15 mM dATP, 20 μl of 15 mM dCTP, 20 μl of 15 mM dTTP, 6 μl of 15 mM dGTP and 80 μCi of [^{3}H]-dGTP specific activity 12 μCi/n mol; Amersham International plc). Twenty units of ribonuclease inhibitor, 5 μl of 10× reverse transcriptase buffer (500 mM Tris-HCl pH 8·3, 500 mM KCl, 100 mM $MgCl_2$, 30 mM dithiothreitol), 6 μl diethylpyrocarbonate

treated water and 3 units RAV-2 (Amersham International plc) were added and primer extension allowed to proceed at 37 °C for 2 h. Unincorporated nucleotides were removed from the cDNA by elution through a column of 5% w/v Sephadex G-50 (Pharmacia, LKB Biotechnology, Uppsala, Sweden).

Polymerase chain reaction amplification of cDNA

PCR amplification of the prepared CVB4 cDNA was attempted on all ten isolates using primers 1 and 2 and on all isolates (except A) using primers 1 and 3. Each reaction mix contained 10 μl cDNA (10 ng), 10 μl of 10 × amplification buffer (500 mM KCl, 100 mM Tris-HCl pH 8·3, 15 mM $MgCl_2$, 0·1% gelatin), 4 μl of each 1·25 mM stock of dATP, dCTP, dGTP and dTTP, 5 μl primer 1 (100 ng/μl), 5 μl of primer 2/3 (100 ng/μl), 2·5 units of Taq DNA polymerase and 53·5 μl of sterile deionized water. Reaction mixes were overlaid with mineral oil and subjected to a 3 min denaturation phase at 94 °C, followed by 30 cycles of 94 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min, and a final extension phase of 72 °C for 10 min. The reaction was carried out in a DNA thermal cycler (Perkin-Elmer Cetus).

Cloning PCR fragments and sequencing of prepared templates

CVB4 cDNA, amplified by PCR, was purified through Sephadex G-50 spin columns, concentrated by ethanol precipitation and digested with the restriction enzymes *Hae* III and *Hinc* II for 2 h at 37 °C. The restriction enzymes were subsequently removed by phenol:chloroform extraction and the purified PCR fragment digests were ligated into the replicative form of M13 mp18 bacteriophage which had been linearized by Sma I digestion and treated with alkaline phosphatase (Amersham International plc). Recombinant M13 vectors with CVB4 inserts were cloned in *Escherichia coli* JM103 cells and single stranded templates prepared according to Maniatis and colleagues [14]. These templates were sequenced by the dideoxynculeotide chain termination method [15]. At least 12 templates were sequenced for each isolate.

Computer analysis of data

Sequence data was recorded, edited and analysed using the Microgenie sequence software programme developed in 1984 by Queen and Korn and distributed by Beckman Instruments Inc.

RESULTS

Utilization of primers 1 and 2 enabled the PCR amplification of a DNA sequence (800–900bp) for isolates A and 2. Subsequent cloning and sequencing of the PCR product of isolate A and isolate 2 determined the nucleic acid sequence spanning the VP1/P2A cleavage site of isolate A between nucleotides 2556 and 3418 but revealed that non-specific amplification had occurred in the case of isolate 2. Primers 1 and 3 allowed the PCR amplification of a 539 base pair fragment, also spanning the VP1/P2A region, between genomic residues 2880 and 3418 for isolates 1–6 and B–D. Subsequent dideoxynucleotide sequencing of the cloned *Hae* III, *Hinc* II digests of the PCR fragments determined the genomic sequence,

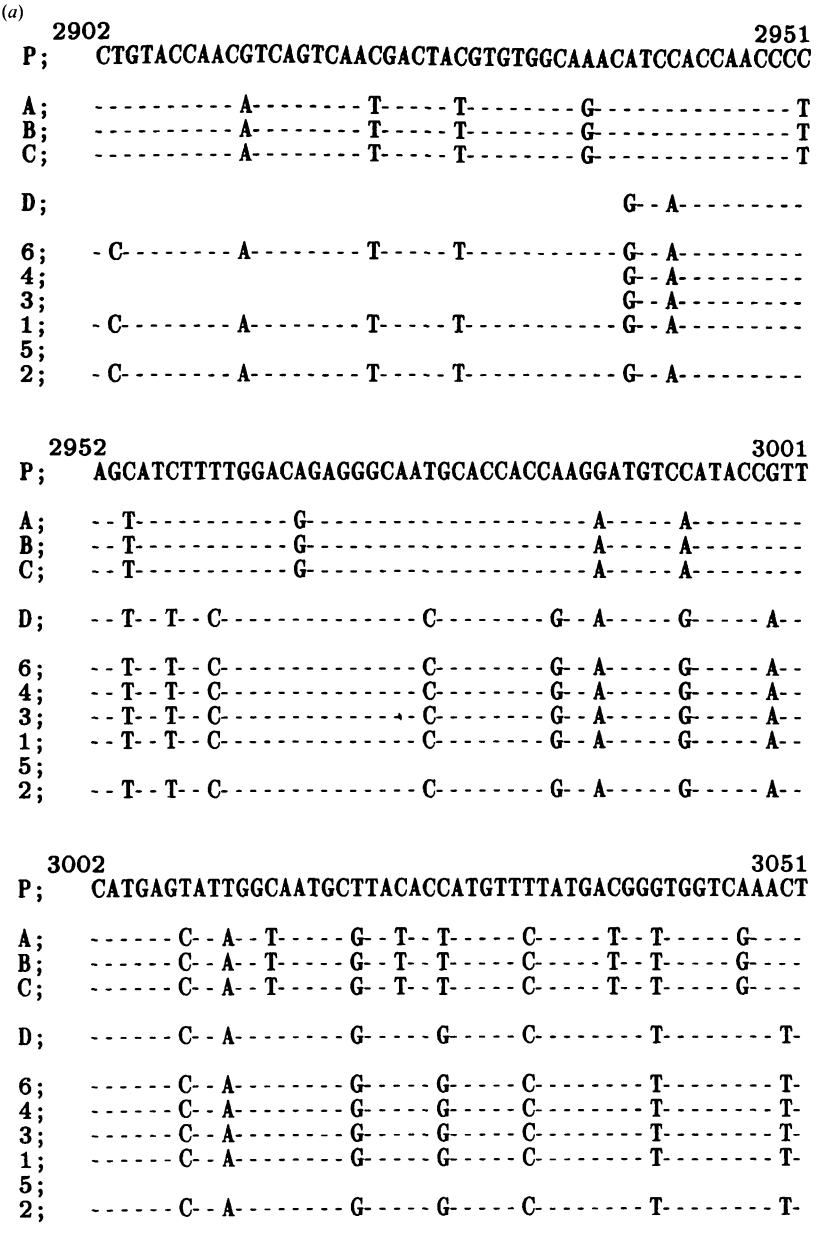

Fig. 1 (*a*) For legend see p. 395.

3120–3228 nucleotides for all nine isolates. In total, a base sequence of 290 nucleotides spanning genomic bases 2939–3228 was established for each of five CVB4 isolates: 1, 2, 3, 4, and D. A 279 base sequence of one isolate; 5, spanning genomic bases 3120–3398 and the complete 497 nucleotide sequence (539bp PCR fragment minus the two primer sequences) for four isolates; A, B, C and 6 were obtained (Fig. 1).

Pairwise comparisons of the nucleic acid sequences of the ten isolates distinguished two distinct groups of CVB4. A, B and C, isolated from clinical

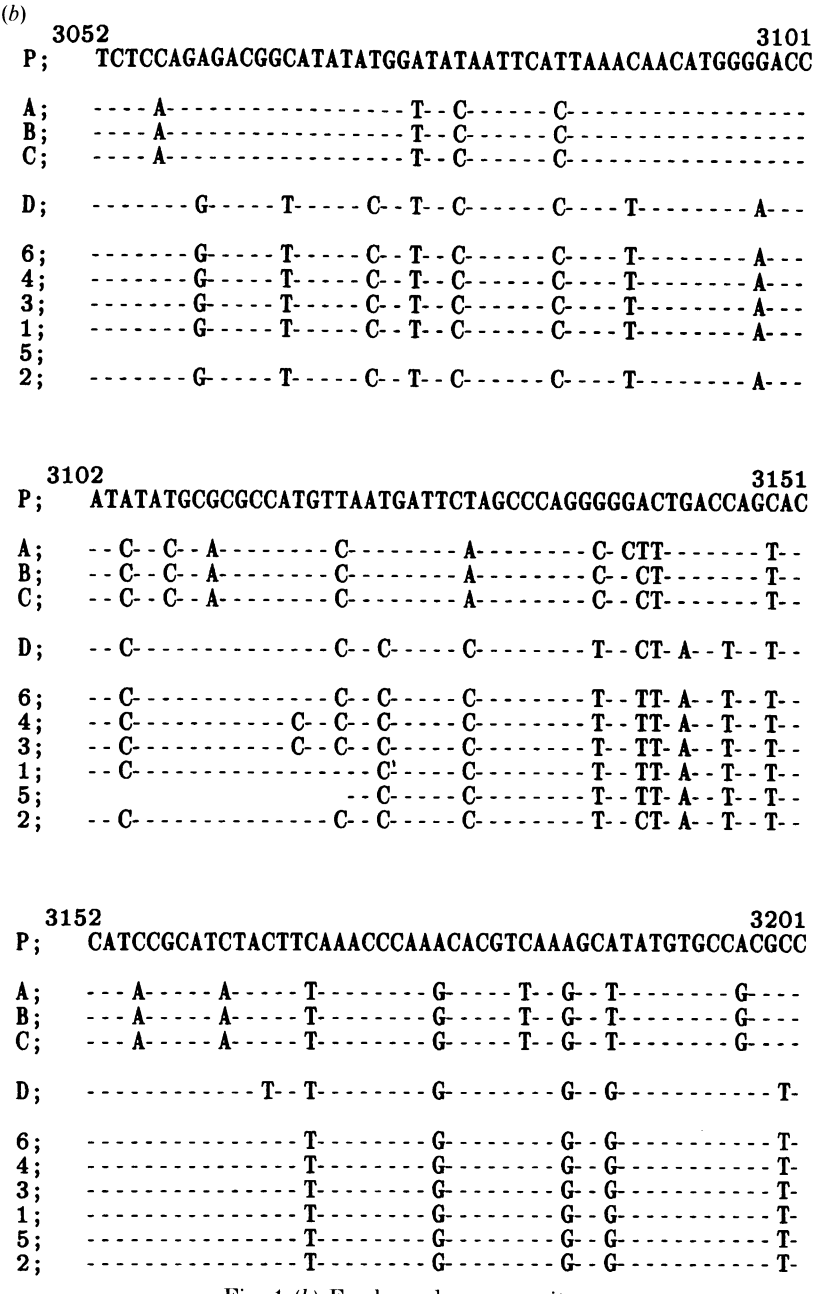

Fig. 1 (*b*) For legend see opposite.

specimens between 1985 and 1986, were classified as group 1. Isolate D, derived from a 1987 clinical specimen, and isolates; 1–6, recovered from environmental samples, between 1986 and 1987, were categorized as group 2. Isolates within each group shared > 99% sequence identity at a nucleotide level. Isolates B and C of group 1 and isolates 3 and 4 of group 2 were identical at a nucleotide level. Between groups, only 80–88% sequence identity was shared depending on whether the VP1 or P2A region of the sequence was compared. All of the isolates

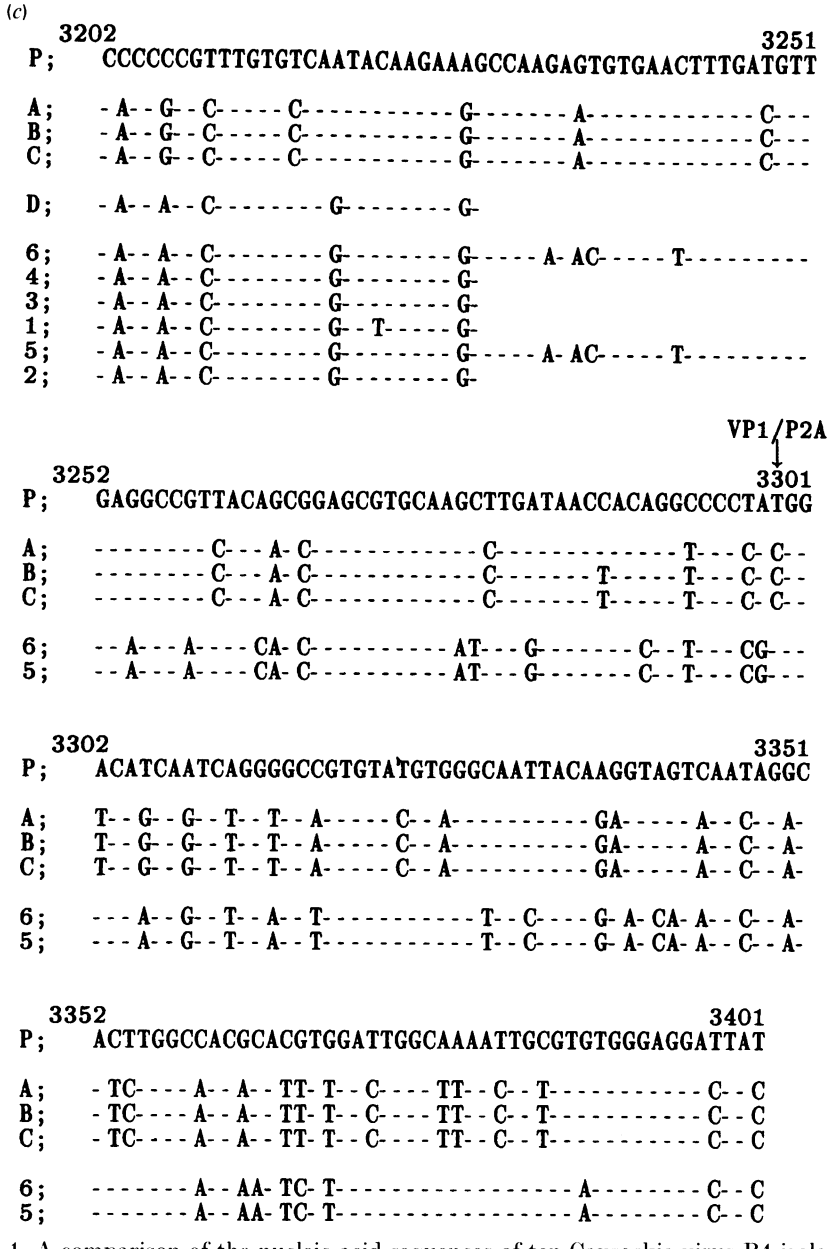

Fig. 1. A comparison of the nucleic acid sequences of ten Coxsackie virus B4 isolates over the region covered by nucleotides 2902–3401. Nucleotide differences from the CVB4 prototype (P) are shown. Dashes (−) indicate no change. The VP1/P2A cleavage site is indicated by an arrow.

shared < 86% sequence identity at the nucleotide level with the prototype CVB4 strain which was isolated in 1950 [12].

All of the nucleotide deviations from the prototype sequence were base substitution mutations with a predominance of transition mutations. Group 1 isolates were associated with a higher level of transversion mutations than group 2 isolates. Transversion mutations also constituted a higher percentage of the

total number of mutations in the P2A region than in the VP1 region, for each isolate sequenced through both regions.

The amino acid sequences predicted from the nucleotide sequences of the CVB4 isolates were more conserved than the actual nucleotide sequences, particularly in the VP1 region. Isolates could not be categorized into two groups on the basis of amino acid sequence identity. Most of the changes in the amino acid sequences occurred around the VP1/P2A junction and the first 46 amino acids of the P2A region. The predicted amino acid sequence for the VP1/P2A cleavage site of the three group 1 isolates was histidine/glycine (H/G) and the two group 2 isolates were characterized by arginine/glycine (R/G).

DISCUSSION

The nucleic acid sequence data generated by this study enabled 10 CVB4 isolates of environmental and clinical origin to be classified into two genotypes, each distinct from the prototype strain. CVB4 isolates recovered from the environment in late 1986–7 could be grouped with a 1987 clinical isolate. This genotype had a widespread geographical distribution within N. Ireland. The second group contained viruses isolated in 1985–6 from clinical specimens originating from two adjacent counties. Isolates 3 and 4 from group 1 and isolate B from group 2 were recovered between October and November 1986 in Co. Armagh, indicating that both genotypes appeared to be prevalent within the same area at approximately the same time. The study could be extended to determine whether different subtypes of CVB4 can really co-exist within a given community or whether one variant is displaced by another introduced from a different geographical region. Temporal patterns of occurrence displayed by non-polio enteroviruses have been noted [16–18] and by CVB serotypes recovered from environmental and clinical specimens [3]. It is feasible that subtypes also exhibit temporal predominance.

The predicted amino acid sequences of the isolates were much more conserved than the actual nucleotide sequences. Prabhakar and colleagues [5] were able to distinguish CVB4 isolates using a panel of monoclonal antibodies but in this study the isolates could not have been differentiated by amino acid sequence differences within the region examined. Rico-Hesse and colleagues [9] also found that predicted amino acid sequences from nucleic acid sequencing studies yielded very few insights into the relationships between wild type 1 polioviruses.

Most of the predicted amino acid changes occurred in the first 46 amino acids of P2A and around the VP1/P2A junction, corresponding with the higher percentage of transversion mutations associated with P2A. These findings suggested that intervals within the 539bp nucleotide fragment were subjected to different selection pressures and that evolution of the viral genome was constrained by the three dimensional structural and functional requirements of the VP1 amino acid sequence.

The predicted amino acid sequence data, however, revealed non-conservation of the tyrosine residue of the VP1/P2A cleavage site Y/G determined by Jenkins and colleagues [12] for the prototype CVB4 strain. The same cleavage site is also characterized by Y/G in the three poliovirus serotypes and human rhinovirus 14.

In this study, tyrosine was replaced by histidine for 3 isolates in group 1 and in 2 isolates belonging to group 2, tyrosine was replaced by arginine. These differences suggest that conservation of the tyrosine residue is not important for recognition of the VP1/P2A cleavage site by the viral protease 2A of CVB4. The importance of the second amino acid for protease recognition could be further evaluated by sequencing several CVB4 isolates through the VP1/P2A region to assess whether other genotypes differ at this cleavage site.

Differentiation of enterovirus variants from the environment and clinical sources is of relevance to epidemiological studies and public health. Although only 10 isolates were analysed in this study, nucleic acid sequencing through the VP1/P2A genomic region enabled identification of two CVB4 genotypes in the community of N. Ireland. This methodology could consequently be of value in demonstrating a potential link between clinical and environmental variants of CVB4 and to tracing the spread of CVB4 infections within a specified community. Since not all variants within a given serotype are expected to display the same level of virulence [19], this epidemiological technique could be applied to an investigation of the relationships between CVB4 variants and tissue tropism.

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