SEQUENCE ANALYSIS OF COXSACKIE A9 VIRUSES ISOLATED DURING 1990-1993 IN CUBA FROM PATIENTS WITH MENINGITIS, MYOCARDITIS AND EPIDEMIC NEUROPATHY

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Recibido en febrero de 1994. Aprobado en julio de 1994

Keywords: Coxsackie, epidemic, evolution, neuropathy, virus.

SUMMARY

Eight strains of Coxsackie A9 (CoxA9) isolated from patients with meningitis or epidemic neuropathy (EN) in a four years period (1990-1993) in Cuba were analyzed by RT-PCR and nucleotide sequencing of the segment containing the junction between VP1 and P2A regions (nucleotides 2985-3431 in the Coxsackie A9 Griggs reference strain). Molecular analysis of the sequences suggested the existence of reservoirs of quasiespecies of rapidly evolving CoxA9, independently of the clinical symptoms associated with the virus isolate. Although the analysis was conducted in a short period of time and with a small segment of the virus genome, it contained the RGD motif that has been involved in receptor recognition, thus playing an important role in virus pathogenesis.

In this region no differences were found between viruses isolated from patients with meningitis or from the cerebrospinal fluid of cases with EN that could account for the differences observed in the behavior and clinical picture produced by these viruses. However, differences in other regions could account for these differences. Considering that the CoxA9 were isolated from few cases of EN and the results reported here, the role of the CoxA9 in the ethiology of the disease could be questioned. Other factors/viruses may be involved in the ethiology of the EN.

RESUMEN

Se analizaron las secuencias de la región VP1-P2A (nucleótidos 2985-3431 en la cepa de referencia Griggs de virus Coxsackie A9) de 8 aislamientos de Coxsackie A9 (CoxA9) obtenidos en Cuba de casos de meningitis y neuropatía epidémica (NE) entre los años 1990-93. El análisis molecular de estas secuencias sugirió la existencia de reservorios de *quasiespecies* de CoxA9 que evolucionan rápidamente, independientemente de los síntomas clínicos asociados con el aislamiento viral. Aunque se analizó un corto período de tiempo y una región relativamente pequeña del genoma viral, esta comprende el motivo RGD supuestamente involucrado en la unión al receptor jugando por tanto un papel esencial en la patogénesis del virus.

En esta región no se encontraron diferencias entre los CoxA9 aislados de casos de meningitis y los obtenidos del liquido cefalorraquídeo de pacientes con NE que puedan a priori justificar las diferencias observadas en el comportamiento y cuadro clínico producido por estos virus. No obstante, diferencias en otras regiones del genoma pudieran ser las responsables de estas diferencias. Considerando que los aislamientos de CoxA9 en pacientes con NE fueron minoritarios y los resultados aquí reportados podemos cuestionar el papel de los CoxA9 en la etiología de la NE y suponer un papel para otros factores y/o virus.

INTRODUCTION

An epidemic of neuropathy producing central and peripheral symptoms appeared in Cuba between 1991 and 1993. The clinical and epidemiological characterization of the disease has been published elsewhere (Llanos *et al.*, 1993). The disease was termed *epidemic neuropathy* (EN) and three forms of clinical picture were identified: purely ocular (optic neuropathy), peripheric neuropathy and mixed cases. About 50 000 cases were diagnosed until October 1993. Different hypothesis about the ethiology of the disease were suggested. Toxic-nutritional and biological factors were argued to be involved in the pathogenesis of the epidemic. The toxic-nutritional hypothesis was supported by laboratory evidences and may play a role as a background conditioning requirement for the disease.

The possibility of an infectious agent (namely a virus) acting in association with other factors was evaluated since the beginning of the epidemic due to some similarities that existed between the Cuban epidemic neuropathy and the epidemic of Subacute Myelo-Optic Neuropathy (SMON) from Japan in which different viruses were isolated, including the Inoue-Melnick virus that was considered as the probable cause, although it was never demonstrated (Inoue *et al.*, 1971).

Two types of viral cytophathic effect (CPE) were observed when the cerebrospinal fluid (CSF) from patients was inoculated into a Vero cell monolayer.

Most of the samples showed a weak, slowly progressing and delayed CPE and some other samples showed an enterovirus-like CPE that was characterized as Coxsackie virus A9 (CoxA9) by a neutralization test with the (LBM) pool of sera and by partial determination of the virus genome sequence (Más *et al.*, 1993; Roca *et al.*, 1994).

In this paper we present the sequence analysis of the VP1-P2A coding region of CoxA9 isolated from patients with EN and from patients with meningitis due to CoxA9. The data represent a molecular analysis of clinical samples isolated from different pathological conditions and enabled preliminary analysis of the degree of variation within the CoxA9 phenotype in a short term period. Despite the differences observed in the clinical pictures, no important differences were found between the EN isolates and the CoxA9 associated with meningitis or myocarditis.

MATERIALS AND METHODS

Virus isolates

CoxA9 strains, isolated from patients with different pathological conditions in Cuba were analyzed. The strains were isolated from patients with meningitis, myocarditis, and epidemic neuropathy (Table 1). The serotype of all viruses was confirmed by neutralization tests using the LBM pool of sera. Viruses were propagated twice in Vero cells prior to analysis.

RNA isolation, cDNA synthesis and Polymerase Chain Reaction

The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as previously described with slight modifications (Rotbart, 1990). Twenty five cm flasks showing complete CPE were freeze and thawed three times. Hundred l aliquots were treated with RNAsin (Promega Corp, Madison, Wisconsin, USA) and viral RNA was extracted by the addition of sodium dodecyl sulphate (SDS) to a final concentration of 2.5% followed by the addition of 1 volume of phenol-chloroform (1:1 mixture).

Extracted RNA was precipitated with 0.3 M sodium acetate, pH 5.3 and 2.5 volumes of cold 100% ethanol during 20 min at -70°C and 30 min at -20°C and finally resuspended in 10μL of double distilled water.

Oligonucleotide primers (OLVP1 5' primer, 5'-AACCCCAGCGTCTTTTGGACAGAGGG-3'; OLP2A 3' primer, 5'-TCTCTGTTATAATTTTCCCACAC-3') were synthesized to amplify the region homologous to the 2945-3460 nucleotides of the sequence of the reference CoxA9 (Griggs) strain (Chang *et al.*, 1989). With these oligonucleotide primers, amplification of viral nucleic acid gave a 516 bp product covering the region encoding the C-terminus of VP1 and the N-terminus of the P2A.

The reverse transcription (RT) was performed with 5 μL of each RNA sample. The following components were added: 40 U of RNasin, 4 mL of reverse transcription reaction buffer (250 mM TrisHCl, pH 8.3, 250 mM KCl, 50 mM $MgCl_2$, 50 mM DTT, 2.5 mM spermidine), 4 μLof 1.25 mM dNTP mixture, 1 μL of the OLP2A downstream primer (20 pmol/L), 10 U of avian reverse transcriptase (Promega) and double-distilled water to complete 20 μL.

The mixture was then incubated for 30 min at 42°C: One quarter of the total cDNA reaction was used for the PCR and the following reagents were added: 5 μL of 10X PCR buffer (500 mM KCl, 100 mM TrisHCl, pH 9, 1% Triton X-100), 3 μL15 mM $MgCl_2$, 8 μL of 1.25 mM dNTP mix, 1 μL of each oligonucleotide primer (50 pmols), 0.2 μL of *T.aquaticus* DNA polymerase (5 U/L; Promega) and double-distilled water to complete 50 μL. The reaction was covered with 100 μL of mineral oil. The amplification cycles were performed in a thermocycler apparatus (Gene Ataq, Pharmacia, Sweden). The first cycle was at 95°C for 3 min and was followed for 40 cycles of 1 min of denaturation (94°C), 1 min annealing at 55°C and 1 min primer extension at 72°C. The last step of primer extension was extended to 12 min.

DNA cloning and sequencing

The PCR-generated DNA fragments were purified by gel electrophoresis with phenol extraction, treated with klenow fragment of DNA polymerase I in the presence of nucleoside triphosphates and ligated into an EcoRV-cut Bluescript plasmid (Stratagene). After transformation of E.coli HB 101 cells, recombinant clones were analyzed by restriction analysis. At least three recombinant clones were obtained from each virus isolate.

The sequence of the recombinant clones was performed with the primers KS and SK (Stratagene) and the complete sequence of both positive and negative strains was obtained for at least two independent clones for each isolate. The double-stranded DNA sequences were performed with Sequenase 2.0 (USB-Biochemicals) using ^{35}S-dATP (Amersham, UK). After electrophoresis, the gels were transferred to filter papers, soaked for 10 min in 10% acetic acid and dried at 80°C under vacuum before autoradiography.

Sequence analysis

A multiple alignment of a segment of 447 bp from 8 isolates and the reference (Griggs) CoxA9 strain was carried out using the TREEALIGN computer program run in a PC 486 Unix server.

A split decomposition analysis was carried out following the procedure described by Dopazo ***et al.*** (1993). We first transformed the sequences into a binary representation assigning R to purines and Y to pyrimidines. Next we calculated Hamming distances using just the segments of the alignment without gaps. The isolation indexes $_{A,B}$ were calculated according to Dopazo *et al.* (1993).

The split decomposition analysis was illustrated in a network that perfectly reflects the calculated Hamming distances.

RESULTS AND DISCUSSION

Eight strains of CoxA9 isolated from different clinical conditions in a four years period in Cuba (Table 1) were analyzed by RT-PCR and nucleotide sequencing of the segment containing the junction between VP1 and P2A regions (figure1). The period from 1990 to 1993 included the period where an epidemic of neuropathy occurred in Cuba. The ethiology of the epidemic has not been elucidated. However, several findings suggested a role for a nutritional deficit, toxic agents and a virus surprisingly found in the CSF of the patients.

Table 1
Characteristics of the virus isolates

STRAIN[a]	AGE[b](years)	CLINICAL DIAGNOSTIC[c]	REGION[d]	SAMPLE[e]	EVOLUTION[f]
453.90	6	meningitis	Sgo. de Cuba	feces	good
138.91	0.5	meningitis myocarditis	Sgo. de Cuba	intestine necropsy	deceased
152.91	15	meningitis	Havana	feces	good
047.92	31	EN (optic neuritis)	Havana	CSF	not good
595.92	15	meningitis	Matanzas	feces	good
231.93	15	EN (optic neuritis)	Havana	CSF	unknown
635.93	1.2	meningitis	Sgo. de Cuba	feces	good
057.93	68	EN (polyneuropathy)	Havana	CSF	unknown

[a]The last two numbers correspond to the year in which the isolate was obtained.
[b]Age of the patient at the moment when the virus isolate was obtained.
[c]Clinical diagnostic of the patient from whom the isolate was obtained.
[d]City in Cuba where the patient was diagnosed.
[e]Clinical sample from which the isolate was obtained.
[f]Clinical evolution of the patient after treatment.

This virus forms Picornavirus-like particles as observed under electron microscopy (Roca *et al.*, 1994; Falcón *et al.*, 1994). Furthermore, few isolates showed an enterovirus-like CPE and were classified as CoxA9 by neutralization and partial nucleotide sequence of the viral genome (Roca *et al.*, 1994).

Although Coxsackie viruses are known to cause severe infections of the central nervous system (Moore, 1982), non of the symptoms found during the EN corresponded to the typical picture observed during Coxsackie virus infections (Moore, 1982; Melnick, 1990). This fact, together with the localization of the virus in the CSF, prompted us to compare the partial nucleotide sequences of the genome of these isolates with the corresponding sequences of the CoxA9 that circulated in Cuba associated with cases of meningitis and myocarditis during the same period.

PCR amplification using the primers OL VP1 and OL P2A yielded a DNA fragment from each of the isolates at the predicted size of 516 bp, the size expected from the sequence of the CoxA9 Griggs strain (Chang *et al.*, 1989).

The nucleotide sequences of the amplified fragments are depicted in figure 1. Comparing with the CoxA9 Griggs strain, few substitutions and deletions were observed together with the loss of one triplet in all strains at positions 3300-3302, four aminoacids before the RGD motif that has been supposed to be involved in receptor recognition (Chang *et al.*, 1989). When compared to each other and to the reference Griggs strain, nucleotide sequence differences in the range of 2% to 23% were observed (figure 2a). All the isolates diverged from the Cox A9 Griggs strain with 19-23% divergency (figure 2a). The relevance of these findings is unclear.

RNA virus populations consist of closely related but not identical genomes, due to the limited fidelity of the replication process (Domingo and Holland, 1988). As has been reported before (Dopazo *et al.*, 1993) these viruses form quasispecies (a mutant spectrum centered around a consensus sequence). For this reason it could be more appropriate to use the split decomposition analysis (Dopazo *et al.*, 1993), which searches not only for tree- like kinship relations but detects as well other forms of evolutionary dynamics.

The diagram obtained after split decomposition is not tree-like (figure 2b-c). This result suggest the existence of quasispecies-like reservoirs of rapidly evolving CoxA9, independently of the clinical symptoms associated with the virus isolate. Since the virus isolates were made in a relatively short period of time (4 years) and the region sequenced represents only a small part of the virus genome, we did no expect to find big differences. However, the sequenced region was selected because it contains variable epitopes and the RGD motif that has been suggested to be involved in receptor recognition, thus playing an important role in the pathogenesis of the virus (Chang *et al.*, 1989). In this region we did not find differences between the CoxA9 isolated from cases of meningitis and those obtained from the CSF of patients with EN that could a priori account for the differences observed in the behavior and clinical picture produced by these viruses.

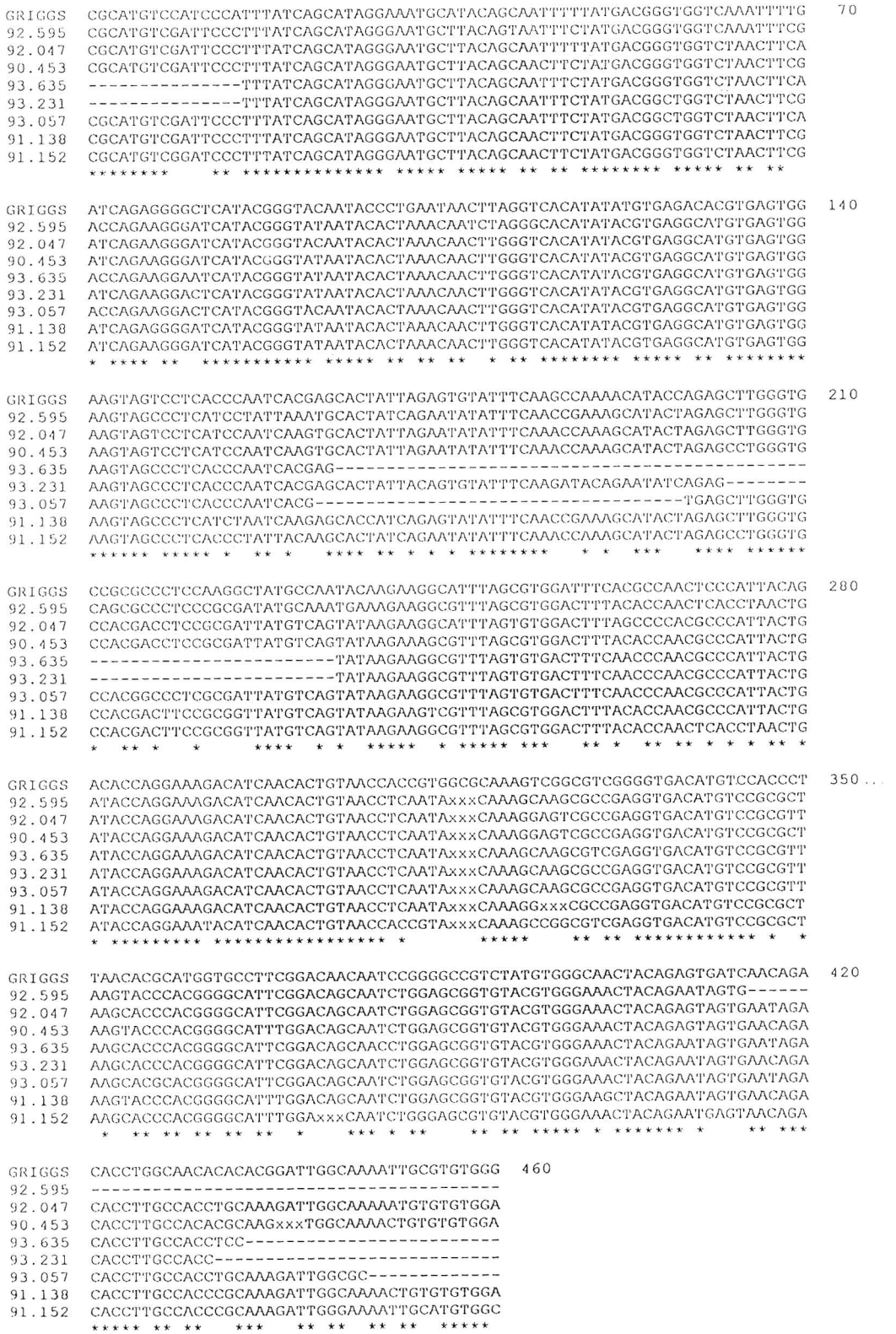
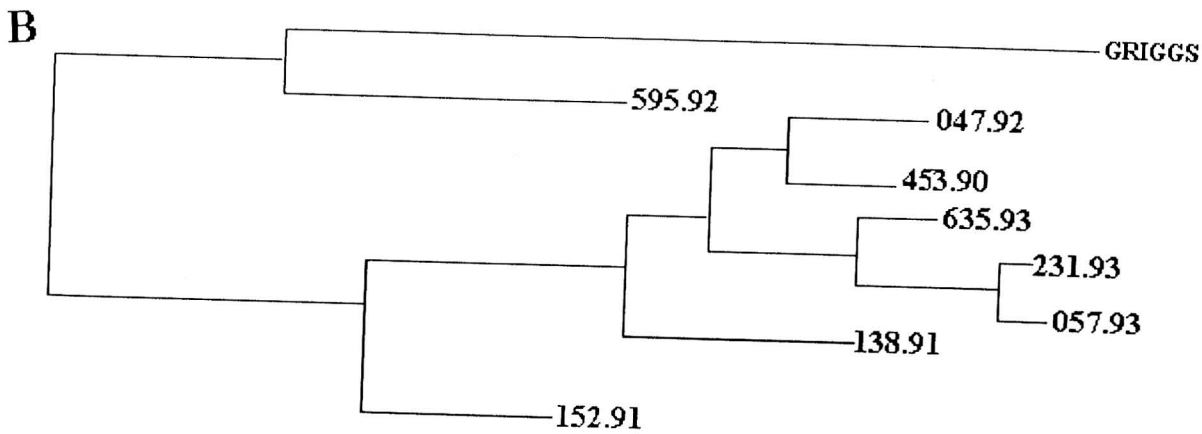


Fig. 1. Alignment of the CoxA9 sequences. Sequences (447 bp; nucleotides 2985-3431 in reference CoxA9 Griggs strain (Chang *et al.*, 1989)) were aligned employing the TREEALIGN computer program. Deletions are denoted by (x) whereas dashed lines (--) denote unknown sequences.

A	1	2	3	4	5	6	7	8
0	78	78	77	81	78	77	77	78
1		94	90	86	94	88	89	91
2			92	86	93	90	91	92
3				84	89	85	87	89
4					84	81	82	84
5						91	91	94



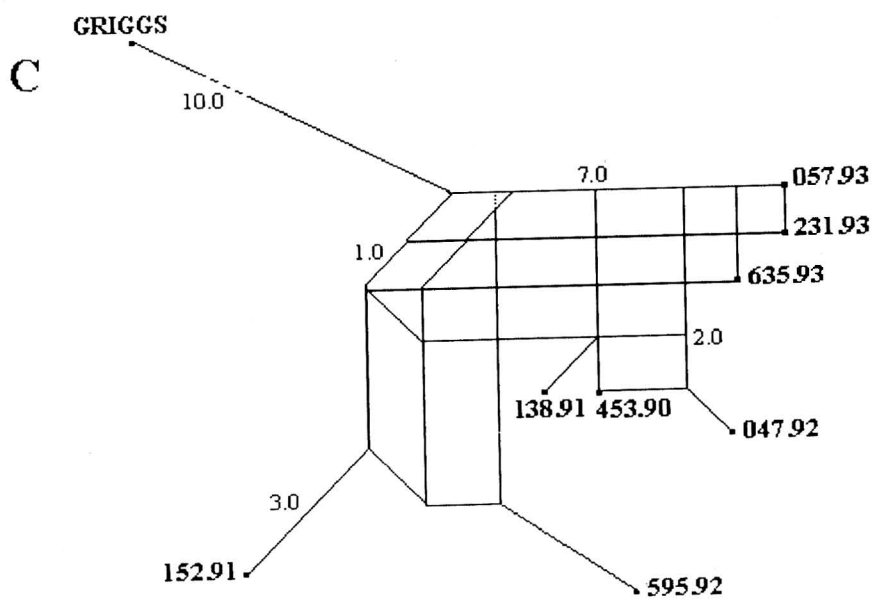


Fig. 2. Sequence analysis of the CoxA9. The relationship between pairs of sequences as percent homology in alignment is shown in (A). In (B), the dendogram obtained after the analysis with the TREEALIGN computer program is shown. (C) shows the decomposition of the metric spaces given by the CoxA9. Virus isolates are numbered as 0: Griggs, 1: 453.90, 2: 138.91, 3: 152.91, 4: 595.92, 5: 047.92 (commonly referred as C-47), 6: 057.93, 7: 231.93, 8: 635.93.

The CoxA9 isolates represented only a small percentage of the isolates since most of them showed the unclassified slow-progressing CPE (Rodríguez *et al.*, unpublished results). Considering this fact and the results reported here, it is tempting to question the role of the CoxA9 in the ethiology of the EN and to propose that other factors/viruses may be involved in the ethiology of the EN. Nevertheless, differences in other regions of the genome may account for the differences observed in the behavior of these viruses.

ACKNOWLEDGEMENTS

The authors will like to thank Dr. P.Más (Institut for Tropical Medicine *Pedro Kourí*, La Habana) for virus isolates, Dr. J.Dopazo (Instituto Nacional de Investigaciones Agrarias, Madrid) for the help with the split decomposition analysis and Dr. R.E.Shope (Yale University School of Medicine) for comments.

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