

**Genus *Coltivirus* (family *Reoviridae*):
genomic and morphologic characterization of Old World
and New World viruses**

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Summary. We report a genomic and morphologic study of the European Eyach (EYA) virus (genus *Coltivirus*, family *Reoviridae*) and a comparative analysis with the American Colorado tick fever (CTF) virus (the type species of the genus). The previously established, but distant, antigenic relationship between these viruses was strengthened by genetic findings (presence of cognate genes, amino acid identity between 55 and 88%, similar conserved terminal motifs, suspected read-through phenomenon in segment 9 of both viruses) and by indistinguishable ultramicroscopic morphologies. Moreover, putative constitutive modifying enzyme activities were suspected to be carried out by homologous viral proteins (RNA-dependent RNA polymerase, methyl/guanylyl transferase, NTPase).

These findings, together with the comparative analysis to genomes of south-east Asian isolates, support the recent classification of arboviruses with 12 segments of dsRNA within two distinct genera (genus *Coltivirus* and genus *Seadornavirus*) and raise interesting questions about the evolutionary origins of coltiviruses. The previously proposed hypothesis that EYA virus was derived from an ancestral virus introduced in Europe with the migration of lagomorphs from North-America, would imply a divergence date between American and European isolates of over 50 million years ago (MYA). This analysis allows for the first time to propose an evolutionary rate for virus dsRNA genomes which was found to be in the order of 10^{-8} to 10^{-9} mutations/nt/year, a rate similar to that of dsDNA genomes.

Introduction

For many years after the discovery of yellow fever virus [36] and the confirmation of Carlos Finlay's earlier finding that the virus is transmitted by mosquitoes, arthropod-borne viruses (arboviruses) were considered to be a singular unique group. It was even supposed that the arboviruses formed a natural taxonomic group [1]. However, in the 1960s and 1970s it became evident that the various arboviruses fall into several taxonomic groups [45]. Among the last of the arboviruses to be characterized and classified were those that are now members of the family *Reoviridae*, that is the member viruses of the genera *Orbivirus*, *Coltivirus* and *Seadornavirus*. This was despite the very early discovery of some member viruses: e.g., the first African horse sickness virus was discovered by M'Fadyean in 1900 [27] and Colorado tick fever virus was discovered by Florio in 1946 [16]. It was not until 1968–1971 that the first biophysical, biochemical and morphological characterization of these viruses permitted their taxonomic placement [9, 33].

Members of the family *Reoviridae* have dsRNA genomes divided into 10 to 12 segments [28]. The member viruses of the genus *Orbivirus* (type species *Bluetongue virus*) have 10 segments, the members of the genus *Coltivirus* (type species *Colorado tick fever virus*) have 12 segments, and the members of the recently identified genus *Seadornavirus* (type species *Banna virus*; previously coltivirus subgroup B) have 12 segments [2, 28]. Among the member viruses of these taxa, the coltiviruses stand out because of their role as human pathogens. The New World virus, *Colorado tick fever virus*, the etiologic agent of Colorado tick fever, is widespread in the Rocky Mountain region of North America. Its distribution matches that of its vector ticks: *Dermacentor andersoni* [15], other *Dermacentor* species, *Ixodes* species, *Haemaphysalis leporispalustris* and *Otobius lagophilus* [13]. The antigenically related Old World virus, *Eyach virus*, was isolated in Europe in 1976 [12, 37] and indirectly incriminated in human neurological disease shortly thereafter [26]. Its distribution matches that of its vector ticks: *Ixodes ricinus* and *Ixodes ventalloi*. Other members of the genus are the California hare coltivirus S6-14-03 from California and the Salmon River virus from Idaho.

The first basis for distinguishing the coltiviruses from other viruses and relating them to each other was serologic cross-reactivity. The viruses exhibit no cross-reactivity with other viruses, but *Colorado tick fever virus* and *Eyach virus* cross-react in complement-fixation tests. The next basis for distinguishing the coltiviruses was virion morphology and morphogenesis. At the time of the first electron microscopic study of *Colorado tick fever virus* in 1968, only a few other arbovirus had been observed to have similar morphology, namely African horse sickness virus and bluetongue virus, two of the founding members of the genus *Orbivirus* which was formalized in 1971 [9, 32, 35, 43]. After *Eyach virus* was isolated in 1976, its morphology was studied cursorily, but until now little has been formally reported.

Since 1997, the use of serology and electron microscopy for taxonomic purposes has declined as knowledge of the molecular biology and genetics of the

viruses has advanced; at first this advance followed upon analysis of the nucleotide sequence of the smallest viral genome segments, then upon that of the entire genome [3]. We present here the complete genomic sequence of the prototype strain of *Eyach virus* and a comparative analysis of the genomic sequences of *Colorado tick fever virus*, additional strains of *Eyach virus*, and California hare coltivirus S6-14-03. We also present here a morphologic and morphogenetic comparison of *Colorado tick fever virus* and *Eyach virus* as a way of extending molecular comparisons to at least on set of phenotypic characters. These analyses have allowed us to consider the close phylogenetic relationships among the member viruses of the genus *Coltivirus* and the more distant relationships among the various genera of the family *Reoviridae*.

Materials and methods

Viruses

Eyach virus (EYA virus) strain Fr577 and EYA virus strain Fr578 were kindly provided by C. Chastel, as lyophilized infected mouse brain. EYA virus strain Gr and California hare coltivirus S6-14-03 (CTFV-S6-14-03) were kindly provided by N. Karabatsos as frozen mouse brain. The Colorado tick fever virus (CTF virus) strain Florio was purchased at the American Type Culture Collection.

Virus propagation

CTF virus strain Florio, EYA virus strain Fr577 and EYA virus strain Fr578 were propagated by intracerebral inoculation of suckling mice. The mice were killed on day 7 post-infection and brains were homogenized in PBS by vortexing with glass beads or prepared for electron microscopy (below). EYA virus strain Gr, obtained as frozen infected mouse brain, was used for genome extraction without further propagation. California hare coltivirus S6-14-03 was adapted to cell culture by 4 passages in BHK-21 cells. The virus was then propagated in BHK-21 cells at 37 °C under 5% CO₂, using Eagle's minimum essential medium supplemented with 2% fetal bovine serum and penicillin G, kanamycin, streptomycin, at 100 IU/ml, 100 µg/ml and 100 µg/ml, respectively.

dsRNA extraction and preparation

CTF virus strain Florio and California hare coltivirus S6-14-03: dsRNAs were extracted from virus infected cell cultures using a guanidinium isothiocyanate procedure (RNA NOW, Biogentex), as described previously [2, 4].

EYA virus strain Fr578: dsRNA was extracted from 5 infected mouse brains after homogenization in phosphate buffered saline using the same RNA NOW method.

EYA virus strains Gr and Fr577: dsRNA was extracted from individual mouse brains using the same method.

Cloning of EYA virus dsRNA

Because EYA virus cannot be grown in cell culture, the sequence of its dsRNA genome segments was obtained by a strategy based on specific stepwise elimination of dsRNA segments (designated the "sequential segment subtraction method", "3SM") [4]. This involves RNase H hydrolysis of specific RNA template segments, after their hybridization

Table 1. Primers used for the amplification of the genomes of CTF virus S6-14-03, EYA virus strain Gr and EYA virus strain Fr577

Primer name	Sequence (5' → 3')	Segment ^a	Map position ^a	Orientation
COL-PLS	GCTGGKGARAGGCATCAAGTWGGTCG	1	1745–1770	sense
COL-PLR	CCAGCSACGAAWCCCATYCCA ACTCC	1	3351–3326	anti-sense
COL6S	CATCCWGAAGCWCAGGARGATGA	6	849–871	sense
COL6R	AATTGTGTATGGCGTGGTGAC	6	2135–2114	anti-sense
COL7S	TGTTGCMAGYCCYKCAAACCKGCTC	7	365–390	sense
COL7R	GCCTCAGCTCCATAWCCAGGACG	7	1861–1839	anti-sense
COL12S	GATGCCCTGYAAYCGCGCTG	12	19–38	sense
COL12R	GACTGCAATTACCCGTCCCGG	12	675–655	anti-sense

^aWith respect to CTFV-FI sequence

to corresponding PCR amplified cDNA. The latter is then removed by DNase treatment. Purification of RNA after each treatment was done using the RNaid kit (Bio 101). The single primer amplification method employed RNA ligase tailing with primer A (5'-PO4-AGGTCTCGTAGACCGTGCACC-NH₂-3') and reverse transcription using primer B (5'-GGTGCACGGTCTACGAGACCT-3') as previously described [4].

*RT-PCR amplification and sequencing of genomic segments 1, 6, 7 and 12
of California hare coltivirus S6-14-03, EYA virus strain Fr577
and EYA virus strain Gr*

The extracted viral dsRNAs from California hare coltivirus S6-14-03, EYA virus strain Fr577 and EYA virus strain Gr were copied into cDNA in the presence of random hexanucleotides and MuMLV superscript reverse transcriptase (Gibco BRL) as described previously [5]. PCR primers were designed from the sequences of the first, sixth, seventh and twelfth genome segments of CTF virus and EYA virus (primer sequences are shown in Table 1). The protocols used for PCR amplification, cloning and sequencing were as described previously [5].

Genomic sequencing of CTF virus strain Florio

The methods used for the cloning and sequencing of CTF virus strain Florio has been described previously [3].

Comparison of polymerase gene sequences

The VP1 sequences of CTF virus strain Florio and EYA virus strain Fr578 were compared with the sequences of putative RNA-dependent RNA polymerases of representative strains of viruses representing eight genera of the family *Reoviridae*. GenBank accession numbers are provided in Table 2.

Sequence analysis methods

All sequence alignments were generated by the Clustal W program [44] and the BLAST program contained in the DNATools program package (version 5.01.661, S.W. Rasmussen). Phylogenetic analyses were performed with the MEGA program [21] using the p-distance determination algorithm. Sequence relatedness was reported as percentage identity or percentage genetic distance. Tree drawing was performed with the Treeview program [34].

Table 2. Sequences used in phylogenetic analysis of the coltivirus; the abbreviations listed are those used in Fig. 3

Species	Isolate	Abbreviation	Accession number
Genus <i>Seadornavirus</i> (12 segments)			
<i>Banna virus</i>	Indonesia-6423	BAV-In6423	AF133430
<i>Kadipiro virus</i>	Java-7075	KDV-Ja7075	AF133429
Genus <i>Coltivirus</i> (12 segments)			
<i>Colorado tick fever virus</i>	CTFV-Florio	CTFV-FI	AF134529
Genus <i>Orthoreovirus</i> (10 segments)			
<i>Mammalian orthoreovirus</i>	Lang strain	MRV-1	M24734
	Jones strain	MRV-2	M31057
	Dearing strain	MRV-3	M31058
Genus <i>Orbivirus</i> (10 segments)			
<i>African horse sickness virus</i>	serotype 9	AHSV-9	U94887
<i>Bluetongue virus</i>	serotype 2	BTV-2	L20508
	serotype 10	BTV-10	X12819
	serotype 11	BTV-11	L20445
	serotype 13	BTV-13	L20446
	serotype 17	BTV-17	L20447
<i>Palyam virus</i>	Chuzan	CHUV	Baa76549
Genus <i>Rotavirus</i> (11 segments)			
<i>Rotavirus A</i>	bovine strain RF	BoRV-A/RF	J04346
	bovine strain UK	BoRV-A/UK	X55444
	simian strain SA11-both	SiRV-A/SA11b	X16830
	simian strain SA11	SiRV-A/SA11	AF015955
	porcine strain Gottfried	PoRV-A/Go	M32805
	avian rotavirus	AvRV-A	Baa24146
<i>Rotavirus B</i>	human/murine strain IDIR	Hu/MuRV-B/IDIR	M97203
<i>Rotavirus C</i>	porcine Cowden strain	PoRV-C/Co	M74216
Genus <i>Fijivirus</i> (10 segments)			
<i>Nilaparvata lugens reovirus</i>	Izumo strain	NLRV-Iz	D49693
Genus <i>Phytoreovirus</i> (10 segments)			
<i>Rice dwarf virus</i>	isolate China	RDV-Ch	U73201
	isolate H	RDV-H	D10222
	isolate A	RDV-A	D90198
Genus <i>Oryzavirus</i> (10 segments)			
<i>Rice ragged stunt virus</i>	Thai strain	RRSV-Th	U66714
Genus <i>Cypovirus</i> (10 segments)			
<i>Bombyx mori cytoplasmic polyhedrosis virus 1</i>	Strain I	Bm-1 CPV	AF323781

Comparison of sequences obtained in this study with those in databases was performed using the NCBI BLAST program (<http://www3.ncbi.nlm.gov/BLAST>). The Pfam program (<http://www.sanger.ac.uk/Pfam/search.shtml>) was used to search for previously described protein family-domains. The Motif program (<http://www.motif.genome.ad.jp>) was used to analyse theoretical protein sequences for the presence of known functional amino acid motifs.

Electron microscopy

Thin section electron microscopy

Brains of suckling mice infected with CTF virus strain Florio were randomly cut into 1 mm³ blocks and processed for sectioning. Tissue was fixed for 60 min in 2.5% glutaraldehyde, postfixed for 30 min in 1% phosphate-buffered osmium tetroxide, dehydrated in a graded ethanol series, and embedded in an Araldite-Epon mixture [30]. Scraped cell monolayers were pelleted by centrifugation at 630 g for 5 min and thereafter processed as brain tissue except for a reduction of glutaraldehyde fixation time to 20 min. Sections were cut with glass knives and stained with lead citrate [37]. Calibration of magnifications was made from photographs of a 54,864 line per inch diffraction grating replica (Ladd Research Industries, Inc., Burlington, Vermont, USA) taken at each magnification step used.

Negative contrast electron microscopy

Carbon-coated grids prepared according to the technique of Simpson and Hauser [41] were rendered hydrophilic by exposure to ultraviolet irradiation. Very small quantities of infected mouse brain tissue were teased apart in large drops of 2% sodium silicotungstate stain, pH 7.0 [17]. Grids were floated on the drops and dried by touching to filter paper. Cell cultures were differentially centrifuged prior to negative staining. Low speed pellets (630 g, 5 min) and ultracentrifuge pellets (100,000 g, 3 h) were dispersed in drops of stain and treated in the same way as brain tissue. Magnification calibration was also performed at the kilovoltage and magnification steps used for photographing negative contrast specimens. Spraying and pseudoreplication techniques were employed but did not yield further resolution of virus structure.

Results

Genomic sequencing

CTF virus genome sequence

The sequence of the 12 segments of the genome of CTF virus strain Florio has been determined: GenBank accession numbers are AF133428, AF139758–AF139764, AF000720, AF139765, U72694 and U53227, respectively for segments 1 to 12.

California hare coltivirus S6-14-03 genome sequence

Partial sequences of segments 1, 6, 7, and 12 of California hare coltivirus S6-14-03, as determined in this study, has been deposited in GenBank: accession numbers are AF343051 (1477 bp), AF343054 (1241 bp), AF343057 (1446 bp) and AF343060 (614 bp).

EYA virus genome sequence

Full-length sequences of segments 1–12 of EYA virus have been determined: GenBank accession numbers are AF282467 to AF282478. The full-length sequence of segment 12 of EYA virus strain Fr578 was found here to be exactly the same as the previously published partial sequence of the same segment [5].

Partial sequences of segments 1, 6, 7, and 12 of EYA virus strain Fr577 and EYA virus strain Gr, as determined in this study, have been deposited in GenBank: accession numbers are AF343052 (1477 bp) and AF343053 (1477 bp), AF343055 (1491 bp) and AF343056 (1491 bp), AF343058 (1241 bp) and AF343059 (1241 bp), AF343061 (617 bp) and AF343062 (617 bp).

Comparison of the genomic sequence of CTF virus and EYA virus

The size of the various segments of CTF and EYA viruses are shown in Table 3. The largest open reading frame (ORF) was determined for each segment. This permitted us to deduce the sizes of the 5'- and 3'-non-coding regions (NCR), and those of the putative proteins encoded by each segment (see Table 3). The proteins encoded by the various dsRNA segments were designated VP_x, where x refers to the number given to the RNA segment, based on its size.

Comparison of the genomic sequence of CTF virus, three strains of EYA virus, California hare coltivirus S6-14-03 and the Seadornaviruses

Genomic sequences of CTF virus strain Florio, California hare coltivirus S6-14-03, EYA virus strain Gr, EYA virus strain Fr577 and EYA virus strain Fr578 were compared with those of viruses which are now recognized as members of the genus *Seadornavirus*. The latter included Banna virus strain BAV-In6423 strain BAV-In6969 strain BAV-In7043 and strain BAV-Ch, and Kadipiro virus strain KDV-Ja7075. These comparisons indicated a maximum of 15% amino acid identity between member viruses of the two genera. This low level of similarity is comparable to values found when comparing viruses in different genera of the family *Reoviridae*. As a frame of reference, the level of amino acid identity between homologous proteins of different viruses within various genera across the family *Reoviridae* is between 20 and 45% [28].

Detailed genome sequence analyses

5'- and 3'-terminal sequences

Analysis of the NCRs of the various genomic dsRNA segments permitted the identification of conserved motifs located at each terminus. In the positive strand of each genome segment of CTF virus strain Florio, the motif [5'-SACUUUUGY-3', where Y = C or U, S = G or C] was found in the 5'-NCR. The motif [5'-WUGCAGUS-3', where W = A or U] was found in the 3'-NCR. The 5'- and 3'-terminal tri-nucleotides of all segments were found to be inverted complements. In all segments except segment 11 the 5'-NCR tri-nucleotide was GAC; in segment 11 it was CAC. In all segments except segment 11 the 3'-NCR

Table 3. Sizes of dsRNA segments 1 to 12 of CTF virus (A) and EYA virus (B), their putative encoded proteins, and their 5'- and 3'-non-coding regions

	Segments size (bp)	Proteins size (AA)	5' NCR		3' NCR	
			Size (bp)	Terminal sequences	Terminal sequences	Size (bp)
A						
Segment 1	4350	1435	13	5'-GACAUUUUGC-----	-----UUGCAGUC-3'	29
Segment 2	3909	1209	45	5'-GACAUUUUGU-----	-----UUGCAGUC-3'	234
Segment 3	3586	1182	11	5'-GACAUUUUGU-----	-----UUGCAGUC-3'	26
Segment 4	3157	1027	21	5'-GACAUUUUGU-----	-----AUGCAGUC-3'	52
Segment 5	2432	751	77	5'-GACAUUUUGU-----	-----AUGCAGUC-3'	99
Segment 6	2141	697	14	5'-GACAUUUUGU-----	-----UUGCAGUC-3'	33
Segment 7	2133	684	23	5'-GACAUUUUGU-----	-----UUGCAGUC-3'	55
Segment 8	2029	660	19	5'-GACAUUUUGU-----	-----UUGCAGUC-3'	27
Segment 9	1884	337 (602)*	40	5'-GACAUUUUGU-----	-----UUGCAGUC-3'	830 (35)*
Segment 10	1880	605	11	5'-GACAUUUUGA-----	-----UUGCAGUC-3'	51
Segment 11	998	249	39	5'-CACAUUUUGU-----	-----AUGCAGUC-3'	209
Segment 12	675	185	19	5'-GACAUUUUGU-----	-----UUGCAGUC-3'	98
			Consensus	5'-sACAUUUUUh-----	-----wUGCAGUs-3'	
B						
Segment 1	4349	1435	12	5'-GACAUUUUG-----	-----UUGCAGUC-3'	29
Segment 2	3934	1275	44	5'-GACAUUUUG-----	-----UUGCAGUC-3'	62
Segment 3	3585	1182	10	5'-GACAUUUUG-----	-----UUGCAGUC-3'	26
Segment 4	3156	1027	20	5'-GACAUUUUG-----	-----UUGCAGUC-3'	52
Segment 5	2432	751	68	5'-GACAAUUU-----	-----AUGCAGUC-3'	74
Segment 6	2178	699	23	5'-GACAAUUU-----	-----UUGCAGUC-3'	55
Segment 7	2139	697	12	5'-GACAUUUU-----	-----UUGCAGUC-3'	33
Segment 8	2028	660	18	5'-GACAUUUUG-----	-----UUGCAGUC-3'	27
Segment 9	1884	337 (602)*	40	5'-GACAUUUU-----	-----UUGCAGUC-3'	830 (35)*
Segment 10	1879	605	10	5'-GACAAUUG-----	-----UUGUAGUC-3'	51
Segment 11	1002	308	39	5'-GACAUUUU-----	-----AUGCAGUC-3'	36
Segment 12	678	184	19	5'-GACAAUUU-----	-----UUGCAGUC-3'	104
			Consensus	5'-GACAwUUK-----	-----wUGyAGUC-3'	

Highly conserved terminal sequences are shown in upper case letters. In consensus sequences, K represents G or U; S represents C or G; W represents A or U and Y represents C or U. *(value) in case of read-through

tri-nucleotide was GUC; in segment 11 it was GUG. In the positive strand of each genome segment of EYA virus strain Fr578, the motif [5'-GACAWUUK-3', where K represents G or U] was found in the 5'-NCR. The motif [5'-WUGYAGUC-3'] was found in the 3'-NCR. The 5'- and 3'-terminal tri-nucleotides of all segments were found to be inverted complements. In all segments the 5'-NCR tri-nucleotide was GAC. In all segments the 3'-NCR tri-nucleotide was GUC that is the same as in CTF virus.

5'- and 3'-terminal sequence motifs

Analysis showed the terminal motifs of the two viruses to be quite similar. The consensus sequence [5'-sACwwUU-3'] was found in the 5'-NCR and [5'-wUGyAGUs-3'] in the 3'-NCR. The conserved terminal sequences of CTF virus and EYA virus although similar, differ in the nucleotide at the fifth position of 5'-NCR of the positive strand (U in case of CTF virus and A or U in the case of EYA virus). A comparison of these sequences to those of member-viruses of different genera of family *Reoviridae* is presented in Table 4. This comparison showed that the last nucleotide at the 3' termini is a cytosine residue for viruses belonging to the different genera of family *Reoviridae* except phytoreoviruses which have a uracil residue. The first nucleotide at the 5' termini is a guanosine residue for viruses belonging to the different genera of family *Reoviridae* except fijiviruses and cypoviruses which have an adenine residue.

Cognate genes

Comparisons of nucleotide and amino acid sequences showed that all of the genome segments of EYA virus have cognate segments in the CTF virus genome (Fig. 1). According to the standard designation of segments in which numbers are assigned in a decreasing order of size (S1 being the largest and S12 the smallest), there is one change in homologous EYA virus and CTF virus segments: segment 6 of EYA virus strain Fr578 is homologous to segment 7 of CTF virus strain Florio and vice versa.

Identification of RNA-dependent RNA polymerase motifs

Motifs found in the polymerase sequences of all members of the family *Reoviridae*, were identified in the VP1 of EYA virus and CTF virus. These are motifs SG (positions 754–755) and GDD (positions 816–818 for CTF virus and 815–817 for EYA virus). The search for sequence homologies using the local BLAST program revealed matches between VP1 of CTF virus and EYA virus (amino acid 500 to 930) and the RNA-dependent RNA polymerases of Nilaparvata lugens reovirus (accession number D49693; identity: 24%, similarity: 39%) and rice ragged stunt virus (accession number U66714; identity: 24%, similarity: 38%). These findings are consistent with the hypothesis that the first genome segments of CTF virus and EYA virus encode the viral RNA-dependent RNA polymerase (VP1(Pol)).

Table 4. Comparison of conserved terminal motifs of member-viruses of genus *Coltivirus* to those of member-viruses of other genera of family *Reoviridae*

Genus	Species (designation)	Consensus sequences	
		5'-NCR	3'-NCR
Coltivirus	Colorado tick fever virus	(CTFV)	5'-G/cACAUUUUG-----A/TUGCAGUG/c-3'
	Eyach virus	(EYAV)	5'-GACAA/TUUG/T-----A/TUGyAGUC-3'
	Banna virus	(BAV) ^a	5'-GUAUA/TA/TAA/TA/TA/TU-----A/GA/CC/TCAG-3'
Seadornavirus	Kadipiro virus	(KDV) ^a	5'-GUAGAAA/TA/TA/TA/T-----A/TA/CC/TCAG-3'
	Bluetongue virus	(BTV) ^a	5'-GUUAAA-----ACUUAC-3'
Orbivirus	Epizootic hemorrhagic disease virus	(EHDV) ^a	5'-GUUAAA-----A/GCUUAC-3'
	African horsesickness virus	(AHSV) ^a	5'-GUUA/UAA/U-----ACA/UUAC-3'
	Broadhaven virus	(BRDV) ^a	5'-GUA AAA-----AA/GGAUAC-3'
Orthoreovirus	Palyam virus	(PALV) ^a	5'-GUA/UAAA-----A/GCUUAC-3'
	St. Croix River virus	(SCRV) ^a	5'-G/AUAAAUA/G-----C/TA/CUAC/T-3'
	Mammalian reovirus	(MRV) ^a	5'-GCUA-----UCAUC-3'
	Avian reovirus	(ARV) ^a	5'-GCUU-----UCAUC-3'
	Nelson bay reovirus	(NBV) ^a	5'-GCUU-----UCAUC-3'
Rotavirus	Baboon reovirus	(BRV) ^a	5'-GUAA-----UCAUC-3'
	Rotavirus A	(RVA) ^a	5'-GGCA/UA/UUA/UAA/UA/U-----A/UUG/UG/UA/GCC-3'
	Grass carp reovirus	(GCRV) ^a	5'-GUUUUU-----UUCAUC-3'
Aquareovirus	Rice ragged stunt virus	(RRSV) ^a	5'-GAUAAA-----GUGC-3'
	Wound tumor virus	(WTV) ^a	5'-GGU/CA-----UGAU-3'
Phytoreovirus	Maize rough dwarf virus	(MRDV) ^a	5'-AAGUUUUU-----UGUC-3'
	Rice black streaked dwarf virus	(RBSDV) ^a	5'-AAGUUUUU-----AGCUNNc/UGUC-3'
	Oat sterile dwarf virus	(OSDV) ^a	5'-AACGAAAA-----UUUUUUUUUAGUC-3'
	Nilaparvata lugens reovirus	(NLRV) ^a	5'-AGU-----GUUGUC-3'
	Cypovirus type 1	(CPV-1) ^a	5'-AGUAAA-----GUUCAGCC-3'
Cypovirus	Cypovirus type 5	(CPV-5) ^a	5'-AGUU-----GAGUUUGC-3'

^aData from Mertens et al. [28]

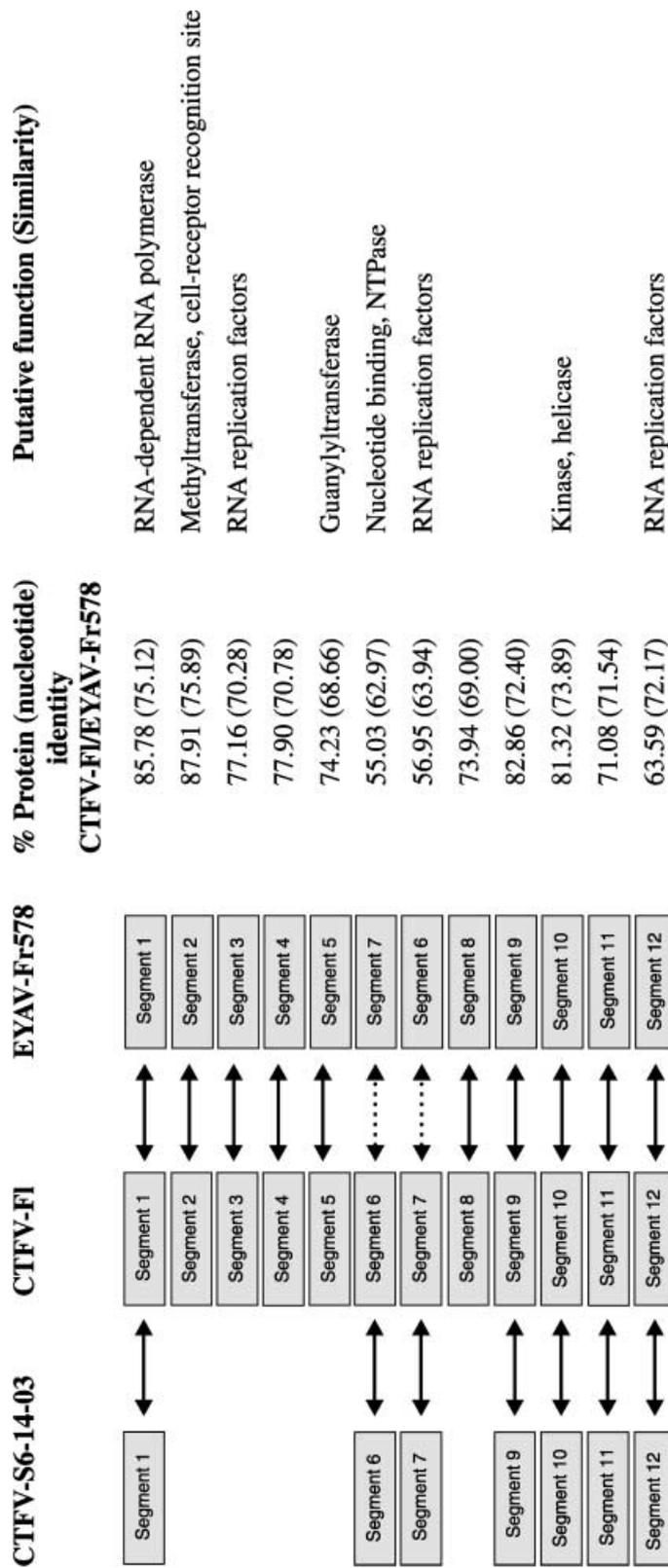


Fig. 1. Comparisons of nucleotide and amino acid sequences of cognate genome segments of EYA virus and CTFV virus

Identification of putative capping enzymes – methyltransferase

Analyses of amino acid sequences using NCBI's BLAST program, showed that amino acid 75–105 of the VP2 of CTF virus and amino acid 75–110 of VP2 of EYA virus matched (identity: 46%, similarity: 53%) a sequence of the DNA adenine methyltransferase of *Chlorella* virus SC-1A (family *Phycodnaviridae*, accession number U65738). The analysis of this region of the *Chlorella* virus DNA methyltransferase showed that it is conserved among a number of DNA methyltransferases.

Identification of putative capping enzymes – guanylyltransferase

Analyses of VP5 showed the presence of a potential phosphamide linkage site (amino acid 342–347 of CTF virus: LNYDKY and for EYA virus: LNYIKH) comparable to that found in the protein encoded by segment 2 of members of the genus *Orthoreovirus* (position 1166–1171: ANPDKF, 40). This protein sequence is thought to be involved in the fixation of guanosine forming the cap structure found on the positive sense strand of orthoreovirus dsRNA.

Identification of putative nucleotide phosphohydrolase enzyme

The NCBI's BLAST program revealed that the region between amino acid 380 and 550 of VP6 of CTF virus resembled a number of nucleotide-binding proteins, such as myosin and kinesin and two distinct purine nucleotide phosphohydrolases (NTPases). These NTPases are the *Archaeoglobus fulgidus* purine NTPase (43% similarity, amino acid 195–356, accession number AE001032) and the *Sulfolobus acidocaldarius* purine NTPase (41% similarity, amino acid 236–408, accession number Y10687). In the homologous EYA virus protein (VP7), the sequence between amino acid 330 and 550 also resembled nucleotide-binding proteins, such as myosin, kinesin and ATPases.

Identification of a putative kinase enzyme

The VP10 sequence of CTF virus and EYA virus contains the pattern [hhhhGx4GKSxnhhhhDD], where h indicates a bulky hydrophobic residue (amino acid 385 to 428) corresponding to a nucleotide-binding site in a number of protein modification enzymes [20, 25]. The local BLAST analysis showed that the region of the protein between amino acid 358 and 589 is homologous to proteins encoded by segment 7 of rice ragged stunt virus (genus *Oryzavirus*, family *Reoviridae*) and Nilaparvarta lugens reovirus (genus *Fijivirus*, family *Reoviridae*). In these latter viruses, segment 7 respectively encodes the non-structural protein NS7, and a core protein which has nucleotide-binding activity. Moreover, in the same region (amino acid 379 to 511), the Pfam analysis also revealed the presence of a kinase domain.

Identification of other known functional motifs – presence of RGD motifs

The RGD sequence (arginine-glycine-aspartic acid) is characteristic of a number of cell binding proteins and in the case of viruses this motif is thought to mediate the binding of viral structures to cellular integrins [6, 39]. Three RGD sequences were identified in the VP1 (amino acid 891–893), VP2 (amino acid 942–944) and VP4 (amino acid 172–174) of CTF virus. All of them are conserved in the homologous EYA virus proteins. RGD motifs in VP1 and VP2 are present at the peaks of a single hydrophilic domain, as shown using a Kyte and Doolittle hydrophathy plot [22]. These RGD sequences may therefore be functional. An additional RGD motif is present in a hydrophilic region of CTF virus VP1 (amino acid 1071–1073) but this site is not conserved in EYA virus VP1.

Identification of other known functional motifs – presence of a GDD motif

GDD motifs are characteristic of amino acid sequences of polymerases. In addition to the VP1 of both CTF virus and EYA virus, two GDD motifs were found to be present in EYA virus VP3 (amino acid 783–785 and 846–848), the second only being conserved with CTF virus. Another GDD sequence was found in the VP4 of CTF virus and EYA virus (amino acid 448–450).

Identification of other known functional motifs – presence of single-strand binding protein (SSBP) site

An amino acid motif homologous to an SSBP site was found in the VP5 of both CTF virus (DMSSWTNDESRAMQLITWKRLAIN, amino acid 599–623) and EYA virus (EVSTWQPDEKAKVMQLVTWKRLATN, amino acid 599–623). SSBPs are known to play an important role in nucleic acids replication [10].

Identification of other known functional motifs – presence of a cyclic-nucleotide-binding domain

In the VP11 of both CTF virus and EYA virus a cyclic-nucleotides binding domain was found between amino acid residues 83 and 106, (iGKRLVSGDITqfVYrqfRGKLEV for CTF virus and iGKRLVSGDVMTfVFrqfRGRTEV for EYA virus, where upper-case letters represent conserved amino acid residues).

Other significant sequence similarities – similarity of CTF virus and EYA virus VP3 and hepatitis C virus NS5 protein

Database searching showed some similarity between the VP3 of CTF virus and EYA virus and the NS5 protein hepatitis C virus, the latter considered as the viral replicase (Accession D49761; amino acid 650–688; identity: 43%, similarity 57%).

Other significant sequence similarities – similarity of VP7 of EYA virus and a protein of the European rabbit

The amino acid sequence (residues 370–490) of the protein encoded by segment 7 (VP7 protein) of EYA virus showed similarities (identity: 24%, similarity: 50%) to the sarcolemmal-associated protein of the European rabbit *Oryctolagus cuniculus* (amino acid A65598.1), the major host of EYA virus [24]. By comparison, the corresponding region of the homologous VP6 of CTF virus showed no match with this rabbit protein.

Other significant sequence similarities – similarities to proteins involved in nucleic acid replication

The VP6 of EYA virus shows considerable divergence from the homologous VP7 of CTF virus, especially between amino acid 267–429 (amino acid for VP7 of CTF virus). However, in the conserved regions of both proteins, matches were identified with nucleic acid replication factors. Between residues 103 and 651 of the CTF virus VP7, partial sequence matches were found with *Arabidopsis thaliana* transcription-elongation factor (61% similarity, accession number AC005499), *Xenopus laevis* VENT-2 transcription factor (43% similarity, accession X98849), bacteriophage RB69 DNA polymerase accessory protein 44 (59% similarity, accession AF039565) and *Synechococcus* sp. RNA polymerase sigma factor (69% similarity, accession U15574). BLAST analysis showed that VP12 of CTF virus and EYA virus is similar to human (P24928), mouse (P08775) and amoebic (T31670) RNA polymerase II (identity: 35–38%, similarity: 45–48%). This sequence (amino acid 113–175) is proline, alanine and serine rich, and is found repetitively in the largest sub-units of these RNA polymerase IIs.

Other significant sequence features – read-through phenomenon

Analysis of the nucleotide sequence of segment 9 of both EYA virus and CTF virus, revealed the presence of an opal stop codon belonging to the class of so-called “leaky” stop codons. This stop codon is flanked at its 3'- end by a cytosine residue, a configuration that has been shown in retroviruses [14] and alphaviruses [42] to allow “read-through.” This phenomenon results from the incorporation of arginine, cysteine or tryptophan in the usual stop codon [14]. Accordingly, two proteins might be synthesized, the first encoded by the ORF between nucleotides 41 and 1054 (ORF1 encoding the protein VP9), the second encoded by the ORF between nucleotides 41 and 1846 (ORF2 encoding VP9'). Comparison of the nucleotide sequences of segment 9 of CTF virus and EYA virus shows that the sequence variation is mainly a function of the third position of the codon. The ORF1 variations at the third position of the codons were calculated using the number of differences estimation method and found to represent 66.4% of the overall variation in this ORF, while the remaining 33.6% were shared by positions 1 and 2. An identical magnitude of variation was found in the part of ORF2 beyond the leaky stop codon. Clearly, this nucleotide variation was not randomly distributed (66.5% at the third position versus 33.4% at positions 1

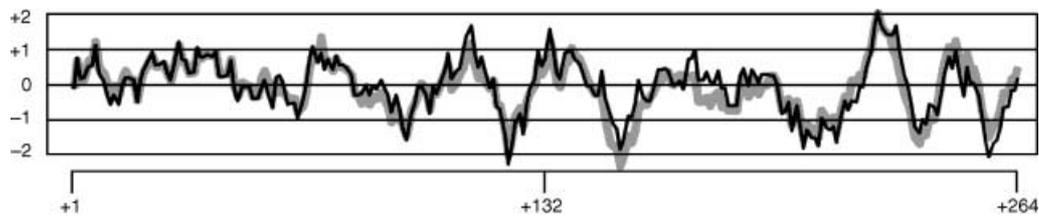


Fig. 2. Kyte and Doolittle hydropathy plots of deduced proteins from the sequence of segment 9 (beyond the suspected leaky stop codon) of CTF virus and EYA virus. The black line represents the hydropathy plot of the deduced protein of segment 9 of CTFV, beyond the suspected leaky stop codon (numbered from +1 to +264, the zero position being attributed to the stop codon). The bold grey line represents the hydropathy plot of the deduced protein of segment 9 of EYAV, beyond the suspected leaky stop codon (numbered from +1 to +264, the zero position being attributed to the stop codon)

and 2, chi square test: $P < 10^{-7}$). This result indicates that this region of segment 9 is submitted to the same kind of genetic selective pressure as regions encoding viral proteins. It is therefore very likely that this region of ORF2 is translated during viral replication. In addition, using the Kyte and Doolittle hydropathy plotting system (with an 11 amino acid sliding window) on the deduced proteins (beyond the suspected leaky stop codon) showed that the putative proteins of CTF and EYA viruses are very similar (Fig. 2). This indicates that the function of the encoded proteins of the two viruses is highly conserved.

Detailed amino acid sequence analyses

Comparison of the amino acid sequence of CTF virus and EYA virus

The amino acid identity between homologous proteins of CTF and EYA viruses ranged from 55 to 88%, with similarity ranging between 72 and 93% (Fig. 2). The most variable proteins are those encoded by segments 6, 7 and 12 with amino acid identities of 55, 57 and 63%, respectively. When sequences of segments 6, 7 and 12 of California hare coltivirus S6-14-03, EYA virus strain Fr577 and EYA virus strain Gr were included in analyses, the same magnitude of variability was observed, as reported in Table 5. In the case of the protein encoded by segment 1 (the viral RNA-dependent RNA polymerase) high identity values were found (86–99%), as seen in Table 5.

Phylogenetic relationships based on amino acid sequences of the viral polymerase

Analyses of the amino acid sequences of the viral polymerase proteins (Pol) of member viruses of particular genera in the family *Reoviridae* indicated relationships that could be used to develop a sense of the overall phylogenetic relationships within the family. There was 21–97% amino acid identity between the various rotaviruses, 97–99% between the various phytoreoviruses, 56–98% between the various orbiviruses, and 92–99% between the various orthoreoviruses. However,

Table 5. The magnitude of variability in the amino acids comprising the homologous proteins of CTF virus and EYA virus

Segment (CTFV/EYAV)	% AA identity ^a (% NA identity) ^a		
	New World coltivirus	Old World coltivirus	New World/Old World coltivirus
1 (homologous to 1 EYAV)	99 (95)	98 (93)	86 (73)
6 (homologous to 7 EYAV)	95 (88)	95 (96)	55 (63)
7 (homologous to 6 EYAV)	93 (94)	95 (93)	56 (64)
12 (homologous to 12 EYAV)	93 (96)	93 (94)	62 (72)

^aMean value

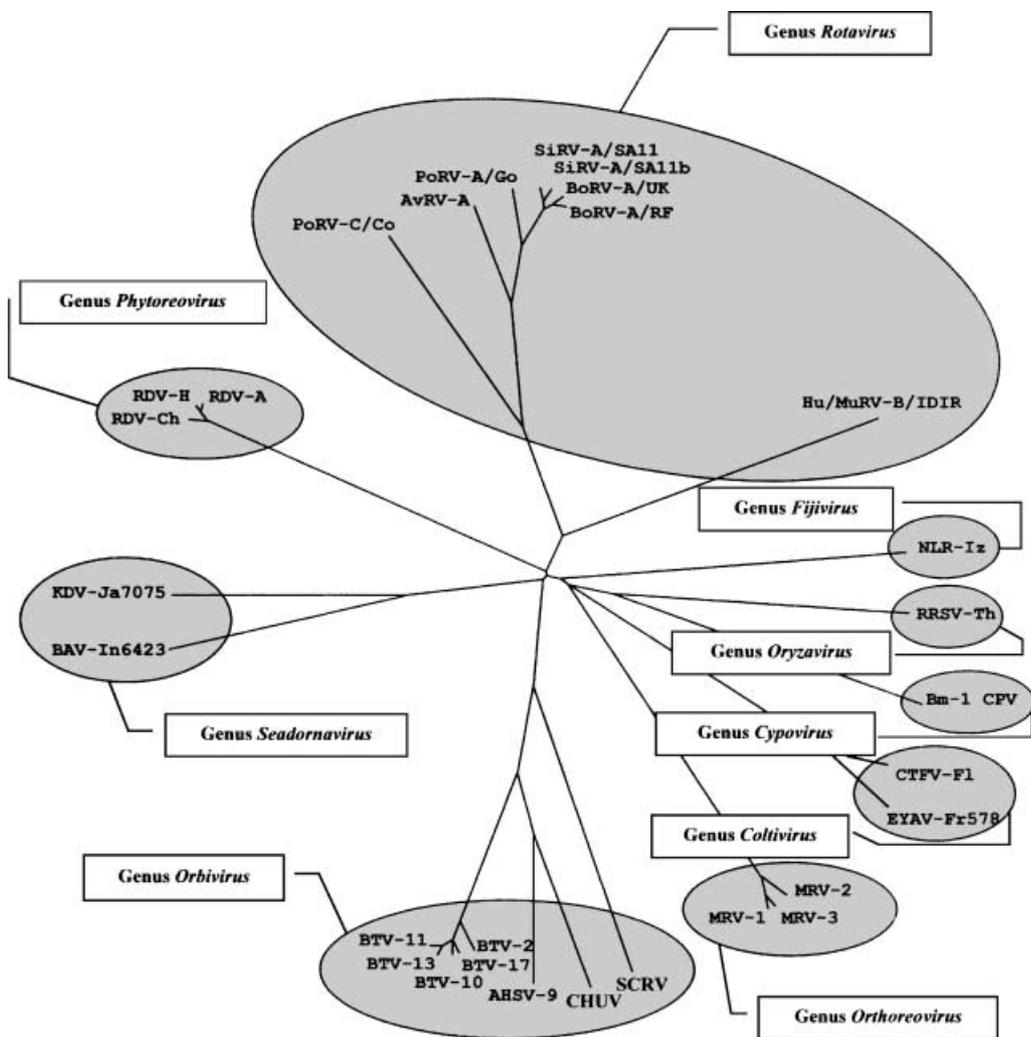


Fig. 3. Viral polymerase [VP1(Pol)] sequence analysis illustrated by a radial neighbor-joining tree

between the various genera, amino acid identity in the polymerase protein was less than 18%.

Within the genus *Coltivirus*, the amino acid identity between CTF virus and EYA virus polymerases is 86%. This contrasts with amino acid identities of only 8.3% and 8.9% between EYA virus and Banna virus and Kadapiro virus, respectively, and 9.6% and 7.8% between CTF virus and Banna virus and Kadapiro virus, respectively. The results of this polymerase [VP1(Pol)] sequence analysis, illustrated by a radial neighbor-joining tree, are displayed in Fig. 3.

Electron microscopy

Thin section electron microscopy

The same progression of events in the maturation of CTF virus and EYA virus was observed in mouse brain and in cell culture. The initial discernible change in cells was the appearance in the cytoplasm of granular areas (identified as viral inclusion bodies, VIB) of moderate electron density. These VIBs had definite yet unbounded margins and some involved large portions of the cytoplasm. Electron dense particles, 45 nm in diameter, were found scattered throughout these granular matrices and especially at their edges; these particles were indistinguishable from cores of progeny virus particles (Figs. 4, 5). The observation of VIBs reveals

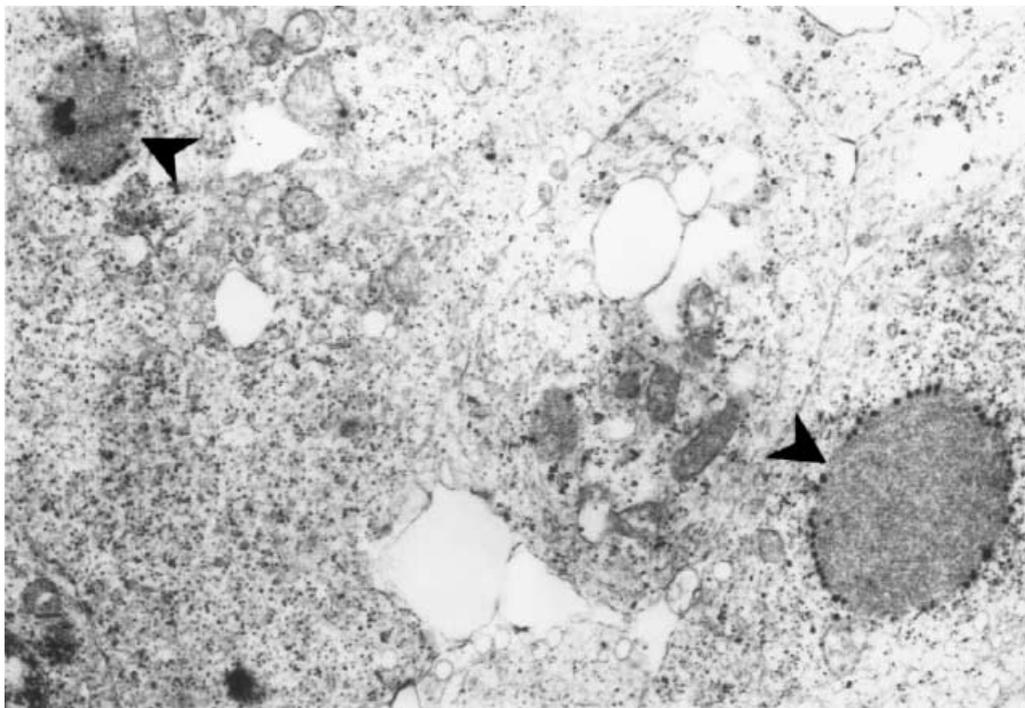


Fig. 4. Cytoplasm of a neuron of a suckling mouse infected 7 days earlier with EYA virus. Infection is marked by the formation of granular matrices or viral inclusion bodies (VIBs) with virions forming at their peripheries. Thin section stained with lead citrate. Magnification $\times 20,000$. The VIBs are indicated by arrows

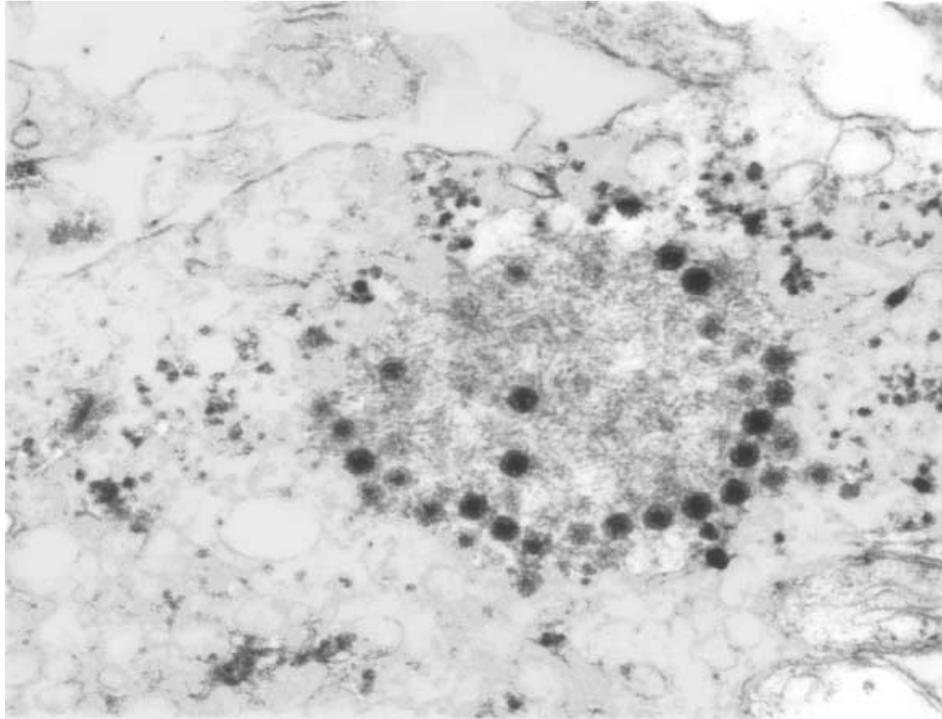


Fig. 5. Higher magnification of a neuron of a suckling mouse infected 7 days earlier with EYA virus. The granular matrices (VIBs) were seen to have definite yet unbounded margins. Virions seem to be in various stages of maturation. Thin section stained with lead citrate. Magnification $\times 60,000$

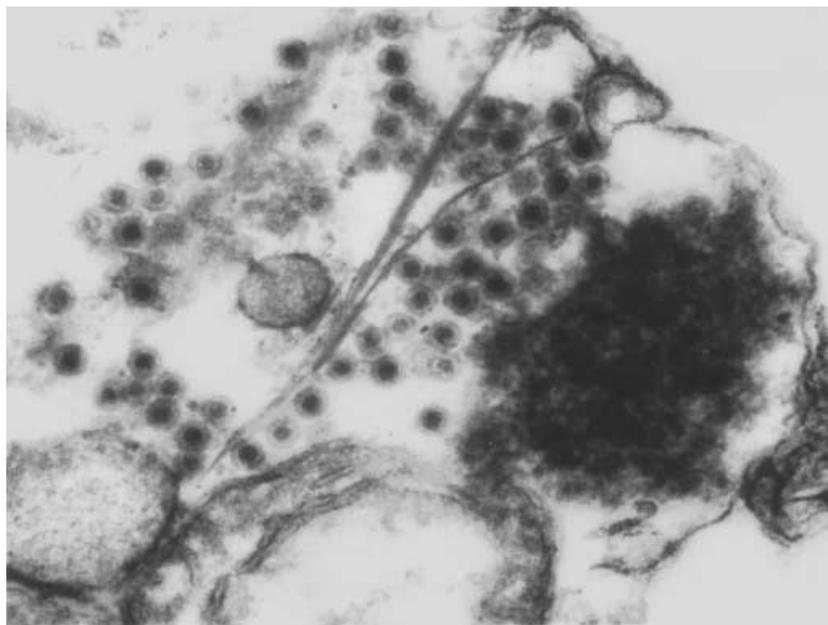


Fig. 6. Rarified cytoplasm of a BHK-21 cell infected with CTF virus. Virions consisted of a homogeneous dense core, 45 nm in diameter, surrounded by an electron lucid layer, considered to be the capsid itself. The total diameter of virions was 75–80 nm. Thin section stained with lead citrate. Magnification $\times 75,000$

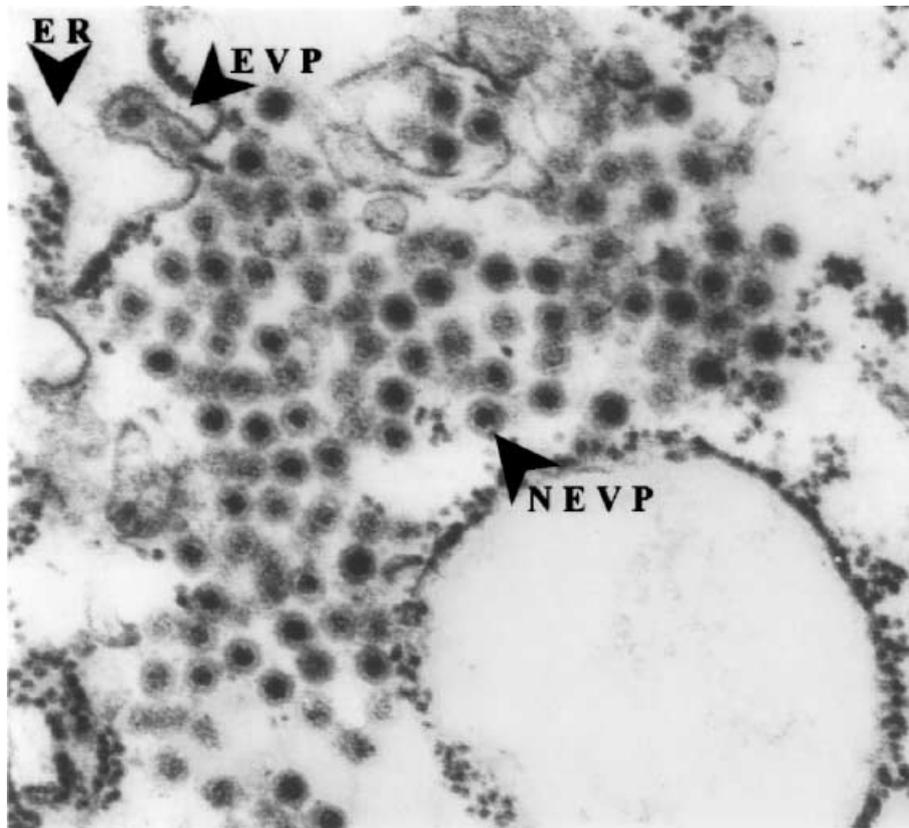


Fig. 7. Cytoplasm of a BHK-21 cell infected with CTF virus. An additional envelope layer was found surrounding some virions as an apparent consequence of passage of capsids through endoplasmic reticulum membranes. Thin section stained with lead citrate. Magnification $\times 60,000$. The enveloped viral particles (E V P), the non-enveloped viral particles (N E V P) and the cisternae of endoplasmic reticulum (E R) are indicated by arrows

possible internal structures that could correspond to early stages of morphogenesis, reinforcing the hypothesis that these granular areas correspond to the site of virus assembly. As infection progressed increasing numbers of virions appeared at the periphery of the granular matrices. Virions consisted of a homogeneous dense core, 45 nm in diameter, surrounded by an electron lucid layer, considered to be the capsid itself. The total diameter of virions was 75–80 nm (Figs. 6, 7). An additional envelope layer was found surrounding some virions located within cisternae of the endoplasmic reticulum. This envelopment appeared to be a consequence of passage of virions through endoplasmic reticulum membranes (Figs. 7, 8). Enveloped particles had a diameter of 90–93 nm. No difference in any morphologic or morphogenetic character was discernable between CTF virus and EYA virus.

With both viruses large numbers of multiple filaments appeared in complex arrays in association with virions and granular matrices (Fig. 9). Arrays of these filaments had cross-striations. These filaments might be equivalent to the “tubules” consisting of the NS1 protein [28], observed in orbivirus infected cells. Within

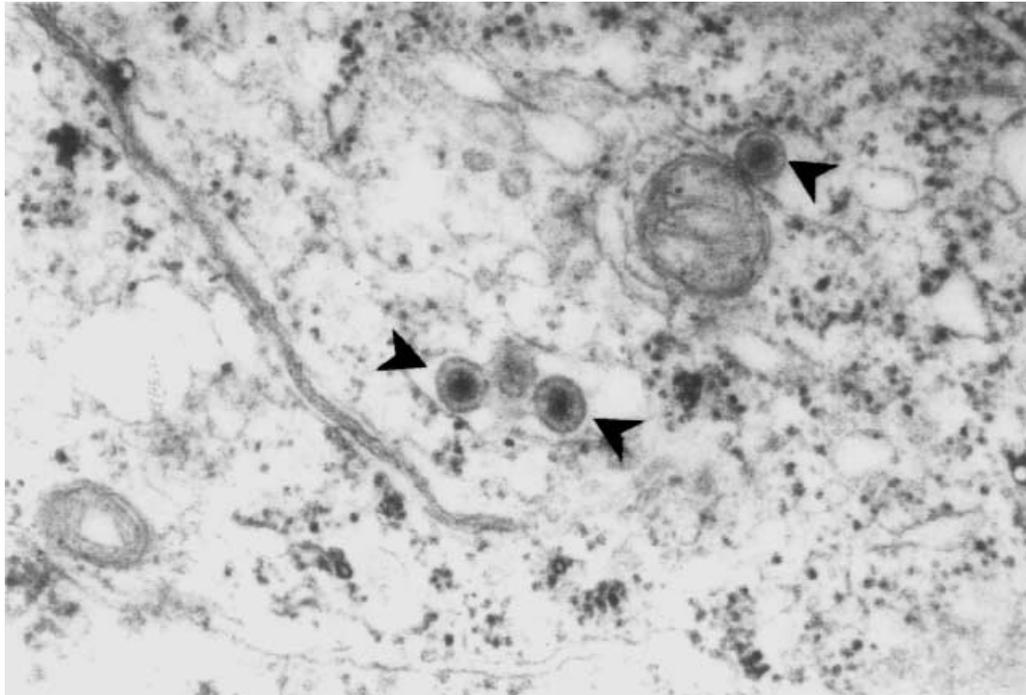


Fig. 8. Cytoplasm of a neuron of a suckling mouse infected 7 days earlier with EYA virus. Individual virions were found within envelopes derived from cytoplasmic membranes. Some viruses, notably coronaviruses, arteriviruses and flaviviruses, migrate in this way to the cell surface and are released by exocytosis – however, we were not able to prove this mechanism of shedding here. Thin section stained with lead citrate. Magnification $\times 75,000$. EYA virions are indicated by arrows

neurons in the brains of suckling mice infected with CTF virus arrays of these filaments occurred within nuclei as well as in cytoplasm. With both viruses, in both suckling mice and cell cultures, relatively few virions were found free in extracellular spaces, even late in infection. Instead, virions accumulated within cells at various stages of degeneration. Virus release appeared to occur via cell lysis.

Negative contrast electron microscopy

CTF virus and EYA virions were observed in negatively stained preparations; they were identical. Virions were round or polygonal in outline with regularly spaced polygonal surface features, which seemed to lie flat on a rather smooth virion surface. Virion mean diameter was 80 nm (range 73–89 nm) (Fig. 10A). Quite prominent in all preparations were delicate round particles 50 nm in diameter (Fig. 10B). These were made up of regular subunits. From observations of partially degraded virus particles and virus particles variably penetrated by stain, and by analogy with the morphological details of orbiviruses and reoviruses, it was concluded that the 50 nm particles represent the virion inner capsid and the 80 nm particles complete virions.

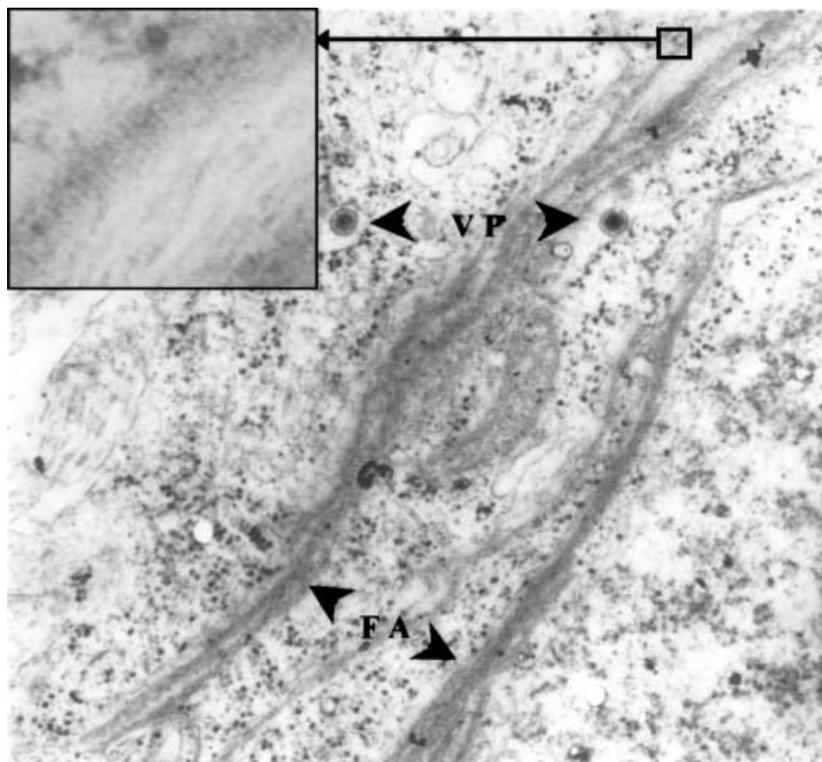


Fig. 9. Rarified cytoplasm of a neuron of a suckling mouse infected 7 days earlier with EYA virus. In cells infected with both EYA virus and CTF virus large numbers of multiple filaments with fine cross-striations appeared in complex arrays in association with virions and granular matrices. Thin section stained with lead citrate. Magnification $\times 40,000$. Viral particles (V P) and filamentous arrays (F A) are indicated by arrows. The cross-striated pattern of the filaments is shown in a higher magnification at the top left part of the figure

As has been observed in the past, the detailed surface structure of CTF virus and EYA virus capsids seemed quite discernable, seemed quite available for analysis of symmetry. However, so few particles have been photographed in a way suitable for such analysis that this has not yet been done.

Discussion

Colorado tick fever virus was identified more than fifty years ago by Florio and colleagues as the etiologic agent of Colorado tick fever, one of the most important tick-borne viral diseases in North America [13]. The virus is the type species of genus *Coltivirus*. In its natural habitat the virus is transmitted between small mammals and the tick *Dermacentor andersoni* and other ixodid ticks [13, 15, 16]. Antigenic variants of the virus have been described; these have been isolated from ticks and vertebrates [8, 19]. Two related but distinct viruses have been identified in North America: one is California hare coltivirus S6-14-03, isolated as the name

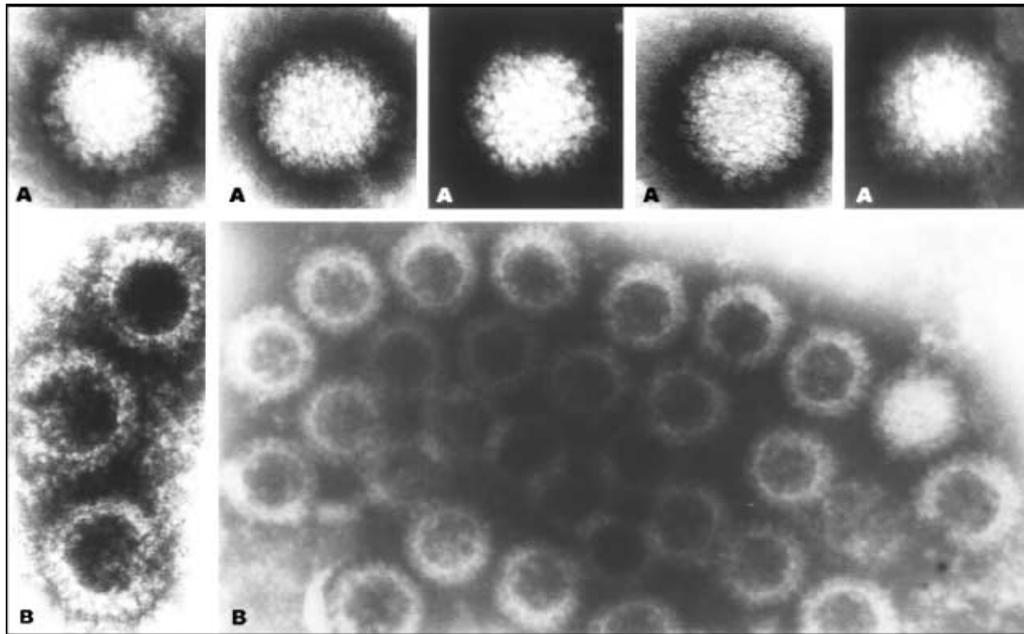


Fig. 10. Negative contrast preparation of CTF virions from infected BHK-21 cell culture. Identical morphology was obtained for EYA virus preparations (data not shown). **A** CTF and EYA virions were round or polygonal in outline with regularly spaced polygonal surface features; however, surface structure symmetry has not yet been analysed. Virion mean diameter is 80 nm. **B** Delicate electron lucent particles (inner capsids), 50 nm in diameter, variably covered with outer capsid subunits. Virus preparation stained with sodium silicotungstate. Magnification of **A** and left image of **B** $\times 200,000$; right image of **B** $\times 150,000$

implies in California from a black-tailed jackrabbit *Lepus californicus* [23]; the other is Salmon River virus, isolated in Idaho from a human patient with a CTF-like illness [31].

A virus related to CTF virus, identified as *Eyach virus*, has been isolated on several occasions in Europe [12, 37]. This virus exhibits a one-way cross-reaction with CTF virus in complement-fixation tests, but each virus has a distinct neutralization profile. More recently, comparison of the sequence of genome segment 12 of CTF virus and EYA virus confirmed this serological relationship [5].

For some years the genus *Coltivirus* was divided into two subgroups, A and B. Subgroup A included CTF, EYA and the other North American and European viruses. Subgroup B comprised viruses isolated from mosquitoes and humans in Southeast Asia. However, recently dsRNA hybridisation analyses, serological analyses and comparison of the genomic sequences of the various viruses led to dropping the A and B subgroup designation and construction of a distinct genus, the genus *Seadornavirus*, for the Asian mosquito-borne viruses [18, 45].

Genetic characterization of the coltiviruses was for some years limited to hybridisation analyses of genomes segments of field isolates of CTF virus; this work only confirmed the narrow range of CTF virus genetic diversity and suggested

possible reassortment in nature of segments 4 and 6 [8]. Recent progress in viral genomics, such as the analyses of the nucleotide sequence of full genomes of the two major coltivirus, as reported here, have permitted a more systematic approach and many new insights into the viruses and their evolutionary relationship.

These sequence analyses have shown that the organization of the genomes of CTF and EYA viruses is similar: (i) the genomes of the viruses are of comparable size (~29,000 bp); (ii) each of the 12 segments of genomic dsRNA of the viruses are of comparable size; (iii) each genome segment is flanked by similar 5' - and 3' - conserved non-coding sequences; and (iv) there is strong evidence that in segment 9 of each virus there is a read-through phenomenon.

These findings are reinforced by the identification, in the cognate genes of the viruses, of sequences encoding similar motifs (functional domains) in non-structural viral proteins as found in certain other viruses and organisms. However, the paucity of available data on coltivirus proteins did not permit clear resolution of many structure-function relationships. An important exception is the unambiguous identification of the viral RNA-dependent RNA polymerase encoded in segment 1 of the viruses. The specific SG and GDD motifs found match polymerase motifs found in the genomes of other member viruses of the family *Reoviridae*. Motifs for protein modifying enzymes including nucleotide phosphohydrolases (NTPases), guanylyltransferases and methyltransferases I and II were also identified. Such constitutive enzymes are known to be involved in capping positive strands of virion RNA segments in other member viruses of the family *Reoviridae*.

Classification criteria within the family *Reoviridae*, based on the quantification of genetic relatedness, have been formally accepted by the International Committee on Taxonomy of Viruses in its Seventh Report [28]. In particular, the degree of relatedness of VP1(pol) amino acid sequences has been accepted as a means of delineating genera and assigning viruses to particular genera. A VP1(pol) amino acid identity of less than 20% has been taken as a criterion for placing viruses in different genera [2]. Accordingly, the comparison of the CTF virus VP1(pol) sequence with that of EYA virus (amino acid identity 86%) unambiguously places the two viruses in the same genus (genus *Coltivirus*). Moreover, on the same basis this genus may be considered distinct from all other genera – the amino acid identity between coltivirus VP1(pol) and all other member viruses of the family *Reoviridae* is less than 20%. In particular, the VP1(pol) amino acid identity between the coltiviruses and the member viruses of the genus *Seadornavirus* (which are also arthropod-borne and also have 12 segment dsRNA genomes) is less than 10% [2].

A number of other molecular and biological characters support the separation of the coltiviruses and seadornaviruses into distinct genera: (i) The genomes of CTF virus and EYA virus are significantly larger than those of Banna virus and Kadipiro virus (~29,000 bp vs. ~20,000 bp); (ii) The G + C content of CTF and EYA virus genomes is 47%, which is significantly higher than the 39% calculated from the full-length genome sequences of Banna virus and Kadipiro virus; (iii) The

conserved terminal sequences of CTF virus and EYA virus are very similar and different from those of Banna virus and Kadipiro virus (which are however very similar to each other); (iv) the natural history of CTF virus and EYA virus involve ixodid ticks (*Dermacentor* and *Ixodes* species), whereas Banna and Kadipiro viruses have been isolated only from mosquitoes (*Culex* species); (v) CTF virus (and related viruses) and EYA virus have been isolated only in North America and Europe, respectively, whereas Banna and Kadipiro viruses have been isolated only in Southeast Asia.

Genetic analyses have indicated that the most important differences between the member viruses of the genus *Coltivirus* are found in the genes encoded in segments 6, 7 and 12 (these segment designations are the same for CTF and EYA viruses). Amino acid identities here are 55%, 56% and 62%, respectively, lower than those seen for other segments of the two viruses. Together with their geographic and ecological isolation and their distant serological relationship, there seems to be a consistent basis for defining the two viruses as distinct species within the genus. The same basis is likely to be useful in the future for the demarcation of other species within the genus.

Of course, two phenotypic characters representing the expression of a large part of the genome of CTF virus and EYA virus are the structure of the virion itself and the complex morphogenetic steps leading to the construction of the virion. In this regard, the authors enjoyed reviewing studies of CTF virus done more than 30 years ago and comparing those observations with unpublished studies of EYA virus. The viruses proved indistinguishable, but there is still more to be done here: the elegant structural studies that have been done in recent years on reoviruses, rotaviruses and orbiviruses (bluetongue virus) using computer analysis of cryoelectron microscopy images and X-ray crystallography should be done on the coltiviruses. The overall similarity in virion structural design of the member viruses of the family *Reoviridae* belies the differences that have evolved as the member viruses of the various genera have occupied such different niches. From the need to withstand a wide pH range and digestive enzymes in the vertebrate intestinal tract and transmission by the fecal-oral route (reoviruses, rotaviruses) to the need to withstand the systemic environment in diverse arthropod vectors and vertebrate hosts and transmission by arthropod bite (orbiviruses), to the need to withstand the unique physiology of plants and plant cells (fijiviruses, phytoreoviruses), very different characters, especially of virion surface structure, are called for. Aspects of the functional qualities of these structures should be addressed by comparative structural studies.

The identification of genetic, serologic and morphologic/morphogenetic relationships between CTF virus and EYA virus raises interesting questions about their evolutionary origin. Several hypotheses have been proposed:

1. It has been hypothesized that EYA virus was derived from CTF virus upon the introduction, in 1953 and 1972, of the hare *Sylvilagus floridanus* into Europe from North America [24]. According to this hypothesis, EYA virus evolved from CTF virus under the selective pressure of adaptation to the European

rabbit, *Oryctolagus cuniculus*, and European ticks. It follows that the observed overall genetic divergence of 25% (and more in segments 6, 7 and 12) occurred over a very short period of time (less than 50 years). This implies a molecular evolutionary rate in the order of 0.5×10^{-2} mutations/nt/year.

2. Alternatively, it has been hypothesized that EYA virus was derived from an ancestral virus introduced into Europe with the migration of ancestors of lagomorphs (hares, rabbits) from North America through Asia [7, 11]. Lagomorph ancestors first appeared during the Eocene epoch (57.8-36.6 MYA) in what was then North America. They are thought to have first migrated into Asia during the Oligocene epoch (34-23 MYA) and by the high Miocene epoch (23-5 MYA) they were common in Europe. This hypothesis implies a molecular evolutionary rate in the order of 10^{-8} to 10^{-9} mutations/nt/year, a rate similar to that of dsDNA genomes.

As a frame of reference, the molecular evolutionary rate of the polymerase gene of tick-borne flaviviruses (single stranded RNA viruses) has been estimated as 5×10^{-5} mutations/nt/year [46]. This rate is based on host divergence of the various viruses between 4,000 and 6,000 years ago.

Even though there is little information on the molecular evolutionary rate of dsRNA viruses, it seems extremely unlikely that the coltivirus could be evolving at a rate consistent with that in the first hypothesis. On the other hand, several lines of thought favor the second hypothesis: (i) It seems reasonable to expect that the stabilizing effect of double-strandedness, as seen with dsDNA genomes, should apply to dsRNA genomes and therefore the molecular evolutionary rate of viruses with such genomes might be expected to approach that of dsDNA genomes (perhaps less the extra stabilizing effect of DNA proofreading enzymes); (ii) It has been proposed that the alternating life cycles of arboviruses in arthropods and vertebrates provides a stabilizing effect – mutations, especially in polymerase genes, that might provide a survival advantage in one host might represent a disadvantage in the other host – so the replication and transmission of the parental type might be favoured overall; (iii) Tick-borne viruses may evolve slower than viruses transmitted by other arthropod vectors or directly from vertebrate host to host – tick vectors remain dormant for long periods during which virus replication cycles must be reduced, and tick vectors remain localized thereby limiting virus radiation and dispersal; (iv) The powerful evolutionary effect of reassortment, so common with dsRNA viruses, may be abrogated by the economic isolation and habits of the tick vectors of the coltivirus – that is, the level of reassortment seen with the other genus of dsRNA viruses, the orbiviruses, might reflect habits and habitats of their *Culicoides* spp. and mosquito vectors rather than the viruses themselves; (v) We know nothing about other coltivirus or similar viruses, viruses that might “fill in the blanks” between CTF virus and EYA virus and provide support for one or another evolutionary hypothesis. For example, it is not inconceivable that another virus might be discovered that would indicate that CTF virus was derived from an EYA-like virus rather than the opposite.

Perhaps the most intriguing aspects of this examination of the various hypotheses for the molecular evolution of the coltivirusess pertain to: (i) the overall molecular evolutionary rate of viral dsRNA genomes in general, and (ii) the more ancient evolutionary history of the divergence of the ancestors of the member viruses of all the genera of the family *Reoviridae* and the other dsRNA viruses.

Another intriguing aspect of this examination of the genomes of the coltivirusess has been the discovery that part of the EYA virus VP7 is clearly distinct from the homologous region of CTF virus VP6 (amino acid identity < 24%), whereas the rest of the proteins are quite similar (amino acid identity > 60%). The distinct region of EYA virus VP7 is similar (~ 50% similarity) to a sarcolemmal-associated protein of the host rabbit in Europe, *Oryctolagus cuniculus*. This raises the question of a possible recombination event between the genome of an ancestor of EYA virus and an mRNA of its lagomorph host. Recombination in dsRNA viruses (distinct from reassortment) has been reported (e.g., in rotaviruses), but only between viral genomes. Recombination between viral and cellular RNAs has been seen in member viruses of the genus *Pestivirus* (family *Flaviviridae*; positive-stranded ssRNA viruses) [29]. Clearly, the significance of the similarity between the EYA virus protein and that of its host warrants further investigation.

The genetic analyses reported here have allowed us to resolve several questions pertaining to the taxonomy of the coltivirusess, but they have raised even more questions. Extending these genetic analyses to a better understanding of the natural history of the coltivirusess will take much more work. One key will be developing a better understanding of the nature and extent of coltivirus variation in nature and a better understanding of the evolution of the various viruses in their complex and geographically and ecologically isolated niches. In this regard, we can hope that more and more coltivirusess will be discovered in nature and characterized in reference laboratories. Another key will be developing a better understanding of the variation in structure-function relationships of the various viral proteins, the architectural organization of the virions, and the nature of variances in viral phenotypes, and pathotypes. Taken together, the morphologic, serologic, biologic, and genetic characteristics that we have studied so far begin to paint the picture of a coherent set of viruses that have solved survival demands in unique fashion. The viruses are entrenched in their econiches and represent in some instances important threats to human health. The viruses are interesting adversaries, worthy of further investigation.

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