Journal of General Virology (1993), **74**, 2753–2757. *Printed in Great Britain*

Co-replication of several isotypes of foot-and-mouth disease virus

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Genome segments of the foot-and-mouth disease virus isolates O_1Lombardy and O_3Venezuela that encode, among other products, capsid protein VP1 were amplified using PCR, and the products were cloned and sequenced. The alignment of up to 11 O_3-specific sequences revealed six silent nucleotide changes as well as six changes that cause amino acid substitutions in capsid protein VP1 at positions 45, 83, 141, 145, 170 and 178. The heterogeneity of three O_1-specific sequences consisted of seven silent exchanges and amino acid changes at positions 85 and 134 on VP1. Amplification, subcloning and sequencing of cloned O_3-specific cDNA was performed to examine the nature of the sequence heterogeneity. As no difference was found among five subcloned sequences, we conclude that the *Taq* polymerase copied the DNA correctly. The sequence heterogeneity observed with both virus isolates is, therefore, consistent with the quasispecies structure of foot-and-mouth disease virus. Furthermore, amino acid changes at a number of sites have been found to be involved in the formation or modulation of neutralizing epitopes. The novel aspect of this study is the ability to estimate, by cloning of PCR products, the number of virus isotypes, possibly varying in antigenicity, that are able to co-propagate. Seven isotypes of O_3Venezuela were identified. Some are of particular interest because they exhibit a change at VP1 codon 145 that causes the replacement of arginine, possibly essential for virus attachment to cells, by isoleucine.

Foot-and-mouth disease virus (FMDV) consists of 60 copies each of the four capsid proteins VP1 to VP4 and one positive-sense single-stranded genomic RNA (Rueckert, 1990). FMDV is extremely variable in antigenicity, and consequently seven serotypes are recognized. Each serotype comprises a number of isolates that differ to a varying extent from each other in immunological reactivity (Pereira, 1977). According to the quasispecies structure of FMDV (Domingo *et al.*, 1992), each virus isolate consists of several genetic and antigenic variants.

From a practical point of view it is important to know about the antigenic variability of an FMDV isolate, because some variants may be better able to resist host defences than others. Antigenic variation is frequently observed at those parts of VP1 which are involved in the formation of neutralizing epitopes (residues 133 to 160 and the C terminus; Strohmaier *et al.*, 1982; Bittle *et al.*, 1982), or that influence steric configuration (residues 40 to 60; Parry *et al.*, 1990). The dominance of VP1 in the structure of the virus surface (Acharya *et al.*, 1989; Logan *et al.*, 1993) explains its importance in antigenicity.

If present, it may be possible to detect sequence heterogeneity among copassaged FMDV by amplifying capsid protein-coding parts of the genome using PCR (Arnheim & Erlich, 1992), followed by molecular cloning of the reaction products and alignment of their sequences.

The suitability of this strategy was examined using the FMDV isolates O_1Lombardy/1946 and O_3Venezuela/1951. One kb of each virus genome was analysed which encodes half of VP3, VP1 and the peptide 2A. As VP1 is the most variable of the FMDV capsid proteins (Palmenberg, 1989), there was a high probability of detecting heterogeneity in the VP1-coding sequence.

Monolayer BHK21 cells (Stoker & Macpherson, 1964) in roller tubes (6×35 cm) were infected either with FMDV O_1Lombardy/1946 (28th passage) or with FMDV O_3Venezuela/1951 (ninth and 11th passages). Ten times-passaged O_3Venezuela was purified from the supernatant of infected cells as described by Strohmaier & Adam (1976). Ten mg virus was obtained, by CsCl gradient centrifugation, from which 1 mg RNA was extracted as described by Marquardt & Adam (1990). Cells infected with O_3Venezuela (which had been passaged 11 times) or with O_1Lombardy were lysed when early cytopathic effect became visible, and total RNA was extracted by the acid guanidinium thiocyanate–

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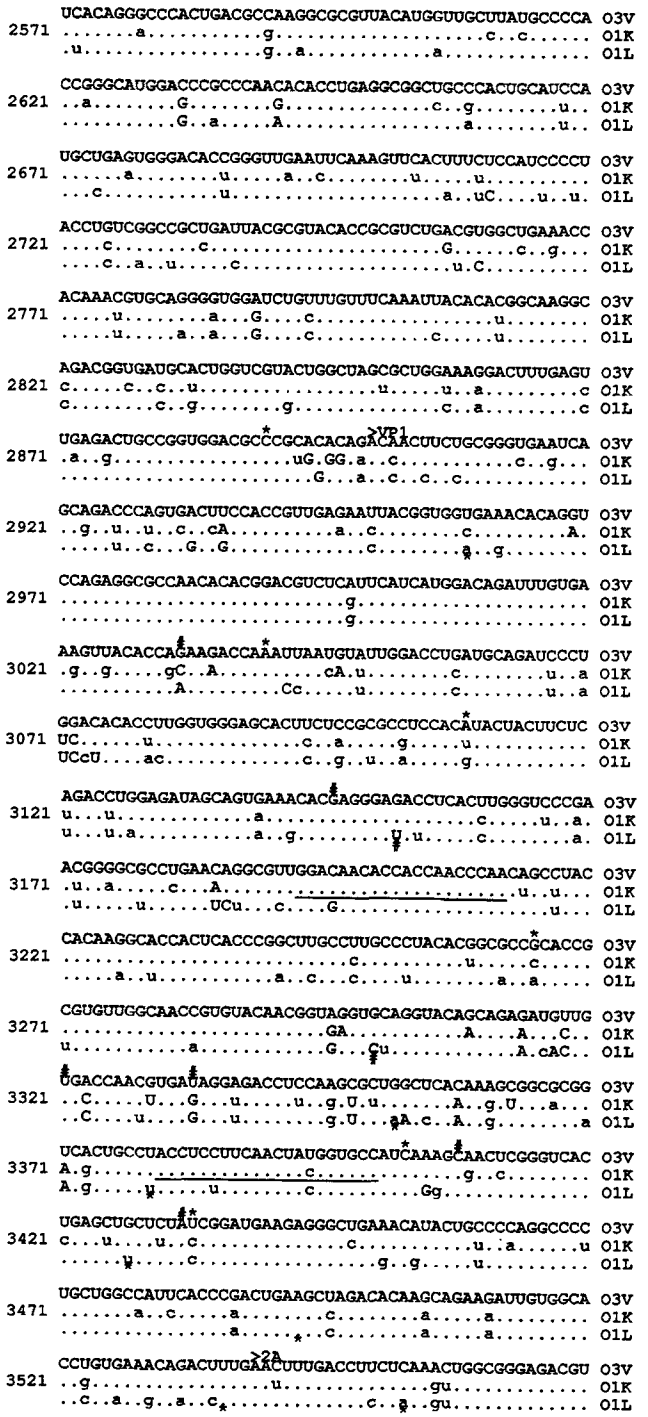

Fig. 1. The O_3Venezuela (O3V) consensus sequence is shown in the top line. Aligned to it are the sequences of O_1Kaufbeuren (O1K), numbered according to the UWGCG data file, and of O_1Lombardy (O1L). Dots indicate sequence identity, lower case letters silent sequence differences, and upper case letters codon changes. >, Beginnings of the VP1- and 2A-encoding regions. Positions causing codon switch among copassaged O_3Venezuela or O_1Lombardy are indicated by # above and below the respective sequence, silent differences by *. Underlining indicates sequences used to bind sequencing primers.

chloroform–phenol method (Chomczynski & Sacchi, 1987).

One μg RNA extracted from virus particles, or 20 μg RNA extracted from FMDV-infected cells, was hybridized with the antisense oligodeoxynucleotide GAAGGGCCCAGGGTTGGACTC, and was then incubated with retroviral reverse transcriptase as recommended by the supplier of the enzyme (Stratagene). Two μl of the reaction volume and 50 ng each of the antisense primer and the positive sense oligodeoxynucleotide CACCATCAACCTGCACTTCA (positions 3572 to 3592 and 2548 to 2567 on the FMDV O_1Kaufbeuren genome map; Forss *et al.*, 1984; UWGCG data file APH01KL.VIRAL) were then subjected to the PCR, performed as recommended by the supplier of the *Taq* polymerase (Promega), in a Bio-med Thermocycler 60. Thirty cycles of polymerization, consisting of denaturation for 1 min at 93 °C, annealing for 2 min at 55 °C and extension for 5 min at 72 °C, were performed. The volume of analytical reactions was 100 μl, that of preparative reactions 1 ml. Occasionally, 10 to 20 ng of the PCR product was amplified by secondary PCR. The yield of PCR products was estimated by comparing reaction aliquots and the DNA ladder (Gibco BRL) in agarose gel electrophoresis, followed by ethidium bromide staining.

Plasmid pSPT18 (Boehringer Mannheim) was linearized at its multiple cloning site by restriction enzyme *Sma*I and dephosphorylated. PCR products were first treated with Klenow enzyme and T4 polynucleotide kinase and then ligated with the vector in a molar ratio of 3:1 according to standard procedures (Maniatis *et al.*, 1982). *Escherichia coli* HB101 cells were transformed with the ligation products and seeded on LB agar dishes. Plasmid DNA was extracted from ampicillin-resistant clones and analysed for inserts by agarose gel electrophoresis.

The FMDV type O specificity of the cloned cDNA was confirmed by sequencing reactions, performed according to the protocol recommended by the supplier of the assay kit (Pharmacia-LKB). The SP6 and T7 promoter primers, as well as the type O FMDV-specific primers mentioned above and indicated in Fig. 1 were used. The FMDV-specific primers were synthesized with an automated DNA synthesizer (Biosearch 8700, Milligen).

Two families of O_3Venezuela-specific cDNA clones were obtained. The first originated from tenfold-passaged, purified virus by primary (family 1.1) and secondary (family 1.2) PCR, the second from virus at its 12th passage by single PCR (family 2). Alignment of sequences obtained from the first clones revealed both changed codons and silent sequence differences. Therefore, further cDNA clones were sequenced. Aligning six sequences, the differences were found to be distributed

Table 1. *Location, nature and frequency of sequence differences among cloned O_3Venezuela-specific PCR products*

Nucleotide position*	Sequence family		
	1.1	1.2	2
2890	ND†	c_1 u_1	c_2 u_2
3032 (045)	ND	A_2	A_3 G_5
3040	ND	a_2	a_7 g_1
3109	ND	a_2	a_7 g_1
3146 (083)	ND	A_2	A_2 G_6
3265	ND	g_1 u_1	g_7
3321 (141)	A_2	A_1 U_1	A_1 U_6
3333 (145)	U_2	U_2	G_3 U_3
3403	c_1 u_1	c_2	c_6
3408 (170)	C_2	C_1 U_1	C_7
3432 (178)	A_2	A_2	A_6 G_1
3433	u_2	c_2	c_1 u_5

* Nucleotides are numbered according to the FMDV O_1Kaufbeuren sequence. Positions of heterogeneous amino acids on VP1 are shown in parentheses, and the crucial nucleotides are indicated by upper case letters. Silent sequence differences are indicated by lower case letters. The number suffixed to each nucleotide indicates the frequency of determination.

† ND, Not determined.

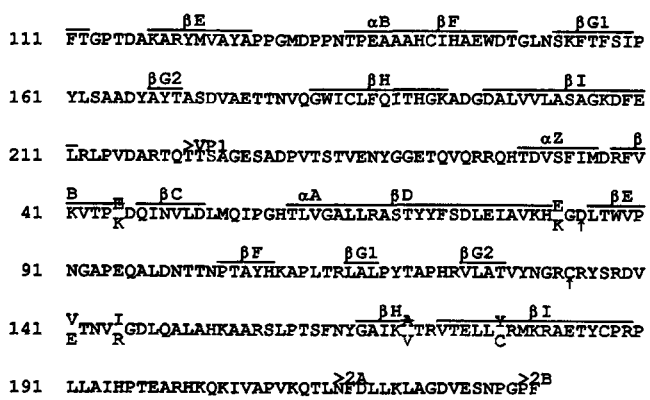

Fig. 2. Sequence, sequence variance and probable structure of capsid protein VP1 of FMDV O_3Venezuela. The amino acid sequence of VP1 and its neighbours of O_3Venezuela, deduced from the nucleotide sequence (Fig. 1), is presented in the one-letter code and numbered. α-helices and β-sheets observed for FMDV O_1BFS are indicated above the sequence, as well as the N-terminal residue (>) of the designated polypeptides. Sequence variation is indicated by two letters at one position. ↑, Positions of sequence variance observed with O_1Lombardy.

unevenly. The VP3-coding part differed at only one position, whereas the VP1-coding part differed at several positions. Therefore, the latter was analysed up to 11 times using both cDNA families.

The alignment of all O_3Venezuela-specific sequences showed six silent nucleotide changes as well as six codon changes (Table 1). All of the latter concerned the VP1-coding region (Fig. 1, 2). An O_3-specific consensus sequence was assembled, and the positions of heterogeneous sequence were marked (Fig. 1). This sequence was aligned to equivalent sequences of FMDV O_1Kaufbeuren, established from cloned cDNA (Forss *et al.*, 1984), and FMDV O_1Lombardy, established either by direct RNA sequencing (Krebs *et al.*, 1991) or by sequencing of cloned PCR products. The alignment showed that O_3Venezuela differed in the sequence under investigation from both other FMDV isolates by more than 13%.

The results presented here were obtained by reverse transcription of FMDV RNA and subsequent *Taq*-dependent DNA polymerization. Together, both reactions can produce nucleotide sequence errors at a rate of about 10^{-4}, which is equivalent to the rate observed during FMDV RNA replication (Domingo *et al.*, 1992). To estimate the error rate in the DNA amplification reaction, one sample of cloned cDNA of O_3Venezuela was subjected to PCR, and the products were cloned following ligation into the vector pBR322, linearized by restriction enzyme *Sca*I. The PCR product sequences of five tetracycline-resistant clones were determined between nucleotide positions 3000 and 3380. No sequence deviation could be detected (data not shown), although this region exhibited seven differences when virus genomes served as templates for cDNA synthesis. It was concluded from these results that the sequence heterogeneity among cloned O_3Venezuela-specific cDNA (Table 1) arose due to genetic heterogeneity of the virus genomes. The finding that there were six positions that differed either in both sequence families, or were found at a different rate in sequence family 2, supports the above conclusion. The other six positions where a different nucleotide was found only once are less certain indicators of heterogeneous virus genome sequences. They may, however, represent minor populations of the quasi-species.

Five of the predicted amino acid changes are located within β-sheet-connecting loops (Fig. 2), protein domains that tolerate amino acid changes more easily than do domains of structural importance. This may be no accident, and might perhaps indicate that the virus was subjected to some kind of selection pressure while circulating in the field, for instance during the course of persistent infection (Gebauer *et al.*, 1988). Support for this hypothesis comes from the fact that sequence heterogeneity of VP1 has been observed at similar positions when monoclonal antibody escape mutants of the FMDV types A, C and O have been sequenced (Thomas *et al.*, 1988; Baxt *et al.*, 1989; Parry *et al.*, 1990; Feigelstock *et al.*, 1992). That antigenic variation occurs in a single passage of FMDV has previously been suggested by the demonstration that virus from different plaques of an isolate differed in immunological reactivity and nucleotide sequence (Rowlands *et al.*, 1983; Mateu *et al.*, 1989).

Table 2. *The observed FMDV O_3Venezuela isotypes*

Cloned cDNA*	Codon number 0451	0831	1411	1451	1701	
1.2.08	AAA	AAG	GAG	AUA	GCA	a†
	K	K	E	I	A	b‡
1.2.10	AAA	AAG	GUG	AUA	GUA	a
	K	K	V	I	V	b
2.21	AAA	AAG	GUG	AUA	GCA	a
	K	K	V	I	A	b
2.03	AAA	AAG	GUG	AGA	GCA	a
	K	K	V	R	A	b
2.15	AAA	GAG	GUG	AGA	GCA	a
	K	E	V	R	A	b
2.06	GAA	GAG	GUG	AGA	GCA	a
	E	E	V	R	A	b
2.11	GAA	GAG	GUG	AUA	GCA	a
	E	E	V	I	A	b

* Digits before the point indicate clone families, others the clone designation.

† a, Codon sequence.

‡ b, Encoded amino acid.

Seven isotypes of VP1 of FMDV O_3Venezuela could be distinguished with regard to the amino acids at positions 45, 83, 141, 145 and 170 (Table 2). As the isotypes differed by up to four residues, it is likely that some may also differ in antigenicity. Further virus isotypes may exist, considering the amino acid change at codon 178 on VP1, and speculating that VP2 and/or VP3 may also exhibit residue changes. No attempt was made to identify all of the isotypes present in passages 9 and 11 of O_3Venezuela, as the results presented here are considered sufficient to demonstrate the coexistence of multiple isotypes. As one virus genome template for cDNA synthesis was extracted from purified virus particles, it is likely that the various protein isotypes assembled correctly to form virus particles.

In order to determine whether different isotypes could also be identified by the same method in other FMDV isolates, a genome fragment of FMDV O_1Lombardy, equivalent to that of O_3Venezuela, was amplified by PCR and cloned. Three cloned PCR products were sequenced. The alignment to the sequence obtained by direct RNA sequencing revealed seven silent differences and heterogeneous codons 85 (encoding either D or Y) and 134 (C or S) in the VP1-coding region (Fig. 1). The corresponding amino acids reside in the loops D–E and G–H. It was concluded that antigenic variation among copassaged FMDV can be identified using the method outlined above, irrespective of the virus isolate under investigation, and that the number of variants thus detected depends upon the amount of sequence analysis performed.

The frequent observation of a codon for Ile instead of Arg at position 145 on VP1 of the O_3Venezuelan genome was unexpected, because Arg is thought to be part of the viral receptor binding site (Fox *et al.*, 1989). The codon for Ile was found in the genome of serially passaged FMDV, indicating the infectivity of the corresponding genome. Whether virus particles that have Ile at position 145 on VP1 are infectious is unknown at present.

The coexistence of isotypes of FMDV has considerable implications for the concept of developing FMDV subunit vaccines (for review, see Brown, 1990), as isotypes may differ in antigenicity. Immunization with antigen of a single homogeneous sequence may not protect against all accumulated virus variants that evolve in the field. The demonstration of coexisting FMDV isotypes by application of the PCR confirms previous observations on antigenic heterogeneity of plaques and escape mutants of FMDV and other RNA viruses (see citations given above, and references therein).

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(Received 23 February 1993; Accepted 19 July 1993)