Molecular epidemiology of foot-and-mouth disease virus type O

Juan Carlos Sáiz, Francisco Sobrino and Joaquín Dopazo

1 Centro de Biología Molecular ‘Severo Ochoa’ (CSIC-UAM), Universidad Autónoma de Madrid, 28049 Cantoblanco and 2 Centro de Investigación en Sanidad Animal, INIA, 28130 Valdeolmos, Madrid, Spain

A phylogenetic tree based on the VP1 sequences of type O foot-and-mouth disease virus (FMDV) has been derived. Direct sequencing of PCR products has been used to obtain the VP1 gene sequences of new isolates. The tree exhibits four main lineages that largely correlate with the geographical origin of isolates. The analysis supports a close relationship between European O1 field isolates and vaccine strains, with the exception of O Thalheim Aus/81 and O Wuppertal Ger/82 which were probably of non-European origin. Analysis of nucleotide substitutions indicates that synonymous mutations play a major role in FMDV evolution.

Foot-and-mouth disease virus (FMDV), an aphthovirus of the picornavirus family, is the causative agent of an economically important disease of cloven-hoofed animals (Pereira, 1981). The genetic and antigenic heterogeneity, as well as the high mutation frequencies, provide FMDV populations with a great potential for virus evolution and adaptability (for reviews, see Domingo et al., 1990; Dopazo et al., 1993a).

The first phylogenetic trees relating FMDVs of types A, O and C were based on nucleotide sequences from the VP1 gene (Dopazo et al., 1988; Palmenberg, 1989). Even though recent results suggest the contribution of capsid proteins other than VP1 to the overall antigenicity of these three serotypes (Barnett et al., 1989; Baxt et al., 1989; Feigelstock et al., 1992; Kitson et al., 1990; Sáiz et al., 1991), the analysis by Dopazo et al. (1988) showed that a VP1-based phylogenetic grouping correlated with the classical serological classification. Likewise, phylogenetic trees based either on VP1 or P1 (VP1, VP2, VP3 and VP4) encoding regions of isolates of serotype C have also shown a good correlation between genetic distances and the subtype classification within this serotype (Martínez et al., 1992).

The incidence of foot-and-mouth disease in Europe has been drastically reduced, mainly due to exhaustive vaccination campaigns and severe control of the outbreaks that have occurred over the last decade (Domingo et al., 1990; Brown, 1993). At the beginning of 1992, a non-vaccination policy was initiated in European countries. However, the potential risk of the reintroduction of the disease, as highlighted by the recent outbreak in Italy, which started in Spring 1993, is not over. A detailed molecular and phylogenetic analysis of viruses causing recent outbreaks in North Africa, the Middle East and South America therefore seems justified (Armstrong et al., 1991). The use of new methods, such as PCR, that can be directly applied to vesicular fluids of putative FMDV-infected animals (Laor et al., 1992; Meyer et al., 1991; Rodriguez et al., 1992), together with the availability of improved sequencing techniques, allows a quick diagnosis of the disease and an epidemiological characterization of the virus.

In the present report we have determined the nucleotide sequences of PCR-amplified VP1 fragments from currently circulating and laboratory strains of FMDV of serotype O, and compared them to previously reported sequences of this serotype (Table 1). Extraction of viral RNA from supernatants of BHK-21 infected cells, reverse transcription, and PCR amplification were basically as described by Rodriguez et al. (1992). The following oligonucleotides were used to prime the cDNA extension: 5' GAAAGGGCCAGGGTGGGACT (complementary to positions 35 to 54 of the 2AB gene) and 5' GATTTGTGAAGGTGACACC (spanning positions 113 to 131 of the VP1 gene). For O Yrigoyen Arg/82 RNA amplification, the latter primer was substituted by 5' GATTACGCGTACACCGCGTC (spanning positions 496 to 515 of the VP3 gene). The primers were chosen using the PCRPROF program (Dopazo et al., 1993b). PCR products were resolved by agarose gel electrophoresis and purified (Magic PCR preps DNA purification system; Promega). Sequencing of PCR products was performed using the fmol DNA sequencing kit (Promega). The following primers for the VP1 gene were used: 5' CGGGTGCGTTTGTGCG (complementary to positions 499 to 516), 5' TTTACGAACTGTTCCAT (complementary to positions 106 to 122), 5' GGGGCTCCCCAGGGTGACC (complementary to
Table 1. FMDV isolates of serotype O used in the present study

<table>
<thead>
<tr>
<th>Virus</th>
<th>Isolation place* and date</th>
<th>Reference</th>
<th>EMBl/GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2 Normandie Fr/49</td>
<td>Normandie (F), 1949</td>
<td>Beck &amp; Strohmaier (1987)</td>
<td>APHVPM</td>
</tr>
<tr>
<td>O2 Brescia It/47</td>
<td>Brescia (I), 1947</td>
<td>Krebs et al. (1991a)</td>
<td>FMDNPCAP</td>
</tr>
<tr>
<td>O1 Lombardy It/46</td>
<td>Lombardy (I), 1946</td>
<td>Krebs et al. (1991b)</td>
<td>APHV12A</td>
</tr>
<tr>
<td>O Wuppertal Ger/82</td>
<td>Wuppertal (D), 1982</td>
<td>Beck &amp; Strohmaier (1987), this work</td>
<td>APHV1L</td>
</tr>
<tr>
<td>O Mateur Tunisia/89</td>
<td>Mateur (TZ), 1989</td>
<td>This work</td>
<td>Z21859‡</td>
</tr>
<tr>
<td>O1 Caseros INTA Arg</td>
<td>Caseros (Arg), NG</td>
<td>Rieder Rojas et al. (1992)</td>
<td>APHVPI</td>
</tr>
<tr>
<td>O1 Caseros INIA Arg/67</td>
<td>Caseros (Arg), 1967</td>
<td>This work</td>
<td>Z21861§</td>
</tr>
<tr>
<td>O1 Ngrodyen Arg/82</td>
<td>Ngrodyen (Arg), 1982</td>
<td>This work</td>
<td>Z21862∥</td>
</tr>
<tr>
<td>O1 Campos I Brazil/58‡‡</td>
<td>Campos (Brazil), 1958</td>
<td>Cheung et al. (1983)</td>
<td>PIVP1OC</td>
</tr>
<tr>
<td>O1 Campos II Brazil/58‡‡</td>
<td>Campos (Brazil), 1958</td>
<td>Jensen &amp; Moore (1993)</td>
<td>APHPVP</td>
</tr>
<tr>
<td>O1 Vac Aus‡‡‡</td>
<td>NG (GB), 1967</td>
<td>Beck &amp; Strohmaier (1987)</td>
<td>APHPV1D</td>
</tr>
<tr>
<td>O1 BFS I UK/67‡‡‡</td>
<td>NG (GB), 1967</td>
<td>Makoff et al. (1982)</td>
<td>PIO1VP</td>
</tr>
<tr>
<td>O1 BFS II UK/67‡‡‡</td>
<td>NG (GB), 1967</td>
<td>Beck &amp; Strohmaier (1987)</td>
<td>APHPV1C</td>
</tr>
<tr>
<td>O1 Funen Dk/82</td>
<td>Funen (DK), 1982</td>
<td>Beck &amp; Strohmaier (1987)</td>
<td>APHPV1H</td>
</tr>
<tr>
<td>O1 Funen Dk/83</td>
<td>Funen (DK), 1983</td>
<td>Beck &amp; Strohmaier (1987)</td>
<td>APHPV1I</td>
</tr>
<tr>
<td>O1 Aulendorf Ger‡</td>
<td>Aulendorf (D), NG</td>
<td>Beck &amp; Strohmaier (1987)</td>
<td>APHPV1B</td>
</tr>
<tr>
<td>O1 Lausanne Sw/65‡</td>
<td>Lausanne (CH), 1965</td>
<td>Beck &amp; Strohmaier (1987)</td>
<td>APHPV1A</td>
</tr>
<tr>
<td>O1 Kaufbeuren Ger/66‡</td>
<td>Kaufbeuren (G), 1966</td>
<td>Kurz et al. (1981)</td>
<td>PIFMDV</td>
</tr>
<tr>
<td>O1 Murchin Ger/82</td>
<td>Murchin (G), 1982</td>
<td>Beck &amp; Strohmaier (1987)</td>
<td>APHPVG</td>
</tr>
<tr>
<td>O1 Wittmar Ger/88</td>
<td>Wittmar (G), 1988</td>
<td>Knowles et al. (1988)</td>
<td>—</td>
</tr>
<tr>
<td>O1 Burgwedel Ger/87</td>
<td>Burgwedel (G), 1987</td>
<td>Knowles et al. (1988)</td>
<td>—</td>
</tr>
</tbody>
</table>

* Country code (international index mark). NG, not given in the reference.
† Corresponding to nucleotides from 139 to 639. No differences with respect to the sequence reported by Beck & Strohmaier (1987) were found in the common fragment from 139 to 300.
‡ Corresponding to nucleotides from 193 to 605.
§ Corresponding to nucleotides from 114 to 481 and from 510 to 639.
∥ Complete VP1 gene.
¶ Vaccine strain.
** The two strains were derived from O1 Campos Brazil/58.
†† The three strains were derived from O1 BFS 1860/UK/67 (Yearbook of Virus Strains used in FMD Vaccines, International Association of Biological Standardization, 1983).

positions 264 to 281) and 5' GCCTACCTCTTCAAC-TACG (spanning positions 477 to 496).

The neighbour-joining method (Saitou & Nei, 1987) was used to derive the phylogenetic trees. To check the reliability of the trees, the bootstrap procedure (Efron, 1982) was used to obtain confidence intervals for branching order (Felsenstein, 1985) and for branch lengths (Dopazo, 1993), as described in Martinez et al. (1992). Following this procedure, a phylogenetic tree for the 24 isolates of FMDV of serotype O (displayed in Table 1) was derived. Since O1 subtype European viruses showed a very close genetic relationship, only two European strains, each representative of a different lineage (see below), were included in the tree shown in Fig. 1. The branching points that were not significant by Felsenstein's classical approach (Felsenstein, 1985) were also not significant when the branch length error analysis was applied. As expected, branches displaying errors of magnitude comparable to their lengths did not statistically support the branching point located at their right on the tree. Thus, the tree displays four main lineages (i to iv in Fig. 1). Three of them correlated with the geographical location: lineage (i) included isolates from South America, and lineages (ii) and (iv) were composed of viruses from Europe. Although not shown in Fig. 1, lineage (ii) also contains an isolate from South America (O1 Campos Brazil/58) whose isolation pre-dates all the others in that group. The two sublineages defined within lineage (i), O1 Caseros INTA Arg and O1 Caseros INIA Arg/67, could be due either to a different isolation date of viruses from the same geographical area or to a different history of laboratory passaging (González et al., 1992). Lineage (iv) comprises O2 subtype viruses as well as the O1 Lombardy It/46 and the O Wien Aus/75 isolates. Lineage (iii) includes viruses from North Africa and the Middle East, as well as two European isolates. Our results strongly support a non-European origin of the O Thalheim Aus/81 and O Wuppertal Ger/82 outbreaks, as suggested by Beck & Strohmaier (1987).

To analyse European O1 subtype viruses further, a more detailed phylogenetic tree including only isolates
Fig. 1. Phylogenetic tree derived from VP1 nucleotide sequences of FMDV isolates of serotype O. For details of the viruses, see Table 1. Vertical distances are arbitrary, but horizontal distances represent the number of nucleotide substitutions per site (n.s.s.). Double lines at the bifurcation points represent the s.o. of the corresponding branch length. (ns) Non-significant branching points; the remainder of the branching points are significant at \( \alpha < 0.05 \). The four different groups showing a high degree of significance, (i) to (iv), are indicated. The point labelled 'o' indicates the root for the phylogenetic tree. The root was situated at a point which makes the phylogeny compatible with the earliest isolation dates, at the middle point between group (iv) and the remaining groups (Martinez et al., 1992).

from group (ii) was constructed (Fig. 2). Two main lineages, A and B, can be distinguished. In both cases a clear relationship can be established between vaccine strains and field isolates. This observation is expected since field isolates have been frequently used to update vaccine strains, which may have been involved in subsequent outbreaks (Beck & Strohmaier, 1987; Carriillo et al., 1990; King et al., 1981). Therefore, the available evidence supports the view that extensive vaccination may modify the pattern of evolution of FMDV (Dopazo et al., 1993a). This may have contributed to difficulties in establishing a correlation between isolation dates and number of accumulated mutations. The position of O1 Campos Brazil/58 (Cheung et al., 1983; Jensen & Moore, 1993), clearly situated among the European O1 isolates, indicates that viral exchanges probably occurred between both continents. The two O1 Campos Brazil/58 strains are sequences derived from the same original isolate. Also O1 BFS isolates and the O1 Vac Aus/75 were derived from the same virus and constitute additional examples of the generation of genetic differences due to laboratory passaging.

Fig. 2. Phylogenetic tree derived from VP1 nucleotide sequences of FMDV type O1 European isolates. Only three branching points, *, were significant at \( \alpha < 0.05 \). In all cases, branch length s.d.s were high and are therefore not represented. Vaccine strains are in italics. The two main lineages, termed A and B, are displayed on the tree. (n.s.s.) Nucleotide substitutions per site.

The number of synonymous (silent) and non-synonymous mutations between pairs of sequences were calculated following the algorithm of Nei & Gojobori (1986). A plot of non-synonymous versus synonymous mutations is shown in Fig. 3. The Pearson correlation coefficient (Sokal & Rohlf, 1981) \( r^2 = 0.219 \), was significantly different from zero \( (t = 3.821 > t_{001}) \), although very low. This low correlation extends to FMDV of serotype O, supporting a recent suggestion by Martinez et al. (1992) that synonymous mutations in FMDV of serotype C play a major role in the evolution of this virus. Strong constraints on the fixation of non-synonymous mutations are the result of at least two
Fig 4. Comparison of the amino acid sequences at the main antigenic sites on VP1 from FMDVs of serotype O. Amino acid sequences, deduced from the nucleotide data, around the antigenic sites 3 (positions 43 to 60; a) and 1 (positions 140 to 160 and 200 to 213; b), according to Kitson et al. (1990) are aligned. Numbering and sequence variation are with reference to isolate O1 Kauffman Ger/66 (Kurz et al., 1981). Dashes represent positions not sequenced. The following amino acid substitutions were found among viruses of the group (ii) not included in the figure: S146 → V in both O1 BFS and O1 Vac Aus; E133 → G in O1 BFS II UK/67; N137 → S in all except for O1 Wettmar Ger/82 and O1 Burgwedel Ger/87; L146 → V in both O1 Campos Brazil/58; A136 → G in O1 Murchin Ger/82.

Factors: (i) these mutations particularly occur at the FMDV flexible residues which can accept changes without disturbing the viral structure (Acharya et al., 1989), and (ii) the tendency is not an accumulation of mutations but alternation at each variable site among a very limited number of amino acids (Martinez et al., 1992). Accordingly, a comparison of the amino acid sequences of the viruses studied indicates that replacements mostly occur at the main antigenic sites on VP1 defined for O type viruses (Kitson et al., 1990) as shown in Fig. 4. In general, a good correlation can be observed between amino acid sequences at these antigenic sites and phylogenetic grouping. With the exception of the presence of O1 Lombardy It/46 within group (iv), this correlation also extends to serological subtyping. As it has been pointed out by Krebs et al. (1991b), this discrepancy is probably due to the C-terminal composition of O1 Lombardy It/46, which is identical to that found for the O2 subtype (see Fig. 4).

The use of a phylogenetic methodology with adequate statistical support has allowed a detailed study of type O FMDV molecular epidemiology and evolution, based on VP1 gene sequences. It can be argued that the use of incomplete VP1 sequences could distort the results. However, an analysis based on the contribution of different regions of the gene for VP1 to the variability of the whole molecule has confirmed the reliability of phylogenies obtained from fragments larger than 250 nucleotides (M.J. Martin, F. Sobrino and J. Dopazo, unpublished). The combination of phylogenetic approaches and the acquisition of sequences from PCR products provides a rapid, accurate and powerful tool for the improvement of epidemiological surveillance.

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