Electrophoretic Properties of Foot-and-mouth Disease Virus Strains and the Selection of Intra-strain Mutants

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SUMMARY

Twenty-six strains of foot-and-mouth disease virus have been studied by sucrose gradient zone electrophoresis and a broad range of mobility is evident. It has been established that the electrophoretic mobilities of strains are a unique heritable property of the virion. No covariation is evident with several in vivo and in vitro characteristics of these strains but there is a relationship between mobility and immunological type. This relationship is unconnected with serological specificity and probably reflects the phylogenetic divergence of the seven immunological types of foot-and-mouth disease virus.

Two types of stable electrophoretic mutant have been isolated by serial selection, one of which, however, exhibits phenotypic instability. The other is stable but can be shown to be at a selective disadvantage in competition with the normal virus.

It has not been possible to demonstrate phenotypic mixing in progeny from cells infected with electrophoretically different strains or mutants. The biological implications of this are discussed.

INTRODUCTION

It is well established that different strains of foot-and-mouth disease virus (FMDV) may exhibit different electrophoretic properties (Poppe & Busch, 1930; Hirtz, 1955; Matheka & Geiss, 1963, 1965a, b; Matheka, Bachrach & Trautman, 1966; Matthaeus, 1966). None the less there has been no comprehensive study of the extent of this variation, nor of its genetic basis. This has been a consequence of either the complexity or the limited resolution of the various techniques employed.

Recently a simplified form of column zone electrophoresis has been described by Thorne (1963) capable of resolving differences between plaque-morphology variants of polyoma virus (Thorne, House & Kisch, 1965) and encephalomyocarditis virus (Breeze & Thorne, 1966). This technique is particularly suited, as in the present case, where comparative measurements are of more value than accurate determination of absolute mobility.

Suitable conditions for the electrophoresis of FMDV by this technique have been determined and the behaviour of 26 strains studied in detail. The extent to which electrophoretic mobility is a heritable characteristic of the virion, the role of the cellular environment and the relationship of electrophoretic properties to a number of in vivo and in vitro marker characteristics have been investigated.

A serial selection procedure, similar to that of Hobom & Braunitzer (1967a,b) with

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phage fd, has been used to isolate electrophoretic mutants of FMDV. Two distinct types of mutant have been obtained. The performance of one of these mutants in competition with the non-mutant has been investigated in a reconstruction experiment.

Phenotypic mixing is a common phenomenon in some picornaviruses (Sprunt et al., 1955; Sprunt, Redman & Alexander, 1959; Ledinko & Hirst, 1961; Dubes & Tolbert, 1961) and furthermore may involve different members of the group (Itoh & Melnick, 1959; Cords & Holland, 1964). By contrast, evidence of phenotypic mixing in FMDV is difficult to obtain (Pringle & Slade, 1968). Electrophoretic variation provides an additional system for the demonstration of phenotypic mixing, and attempts have been made to detect phenotypic mixing in this way.

**METHODS**

**FMDV strains.** The following strains of FMDV were used. They are listed according to immunological type with details of their laboratory histories in terms of number of passages in secondary pig-kidney (PK); baby-hamster-kidney 21, clone 13 (BHK) cells (Macpherson & Stoker, 1962), or IB-RS-2 pig-kidney (RS) cells (de Castro, 1964). Cloned strains had undergone at least three isolations from single plaques.

- **A strains:** IRAQ 24/64, PK10+BHK 1, uncloned; A-119, PK 20+BHK 1, cloned.
- **C strains:** IRAQ 24/64, PK10+BHK 1, uncloned; C-NORVILLE, BHK 6, uncloned.
- **O strains:** ISRAEL 1/63, BHK 108, cloned; OVI, BHK 62, cloned; O LOMBARDY, RS 2+BHK 1, uncloned; O NORTHUMBERLAND, RS 2+BHK 1, uncloned; O STRATFORD, RS 2+BHK 1, uncloned; O CAMPOS, RS 2+BHK 1, uncloned; O LAUSANNE, RS 2+BHK 1, uncloned; KENYA 102/60, BHK 3, uncloned; M 11/52, PK 25+BHK 1, cloned; BFS 1860, BHK 7, uncloned; O FARHAM, RS 2+BHK 1, uncloned.
- **SAT 1 strains:** RV 11/37 (attenuated strain) adult mouse 28+PK 6+BHK 2, cloned; TURKEY 43/62, BHK 11, cloned.
- **SAT 2 strains:** KENYA 40/66, BHK 6+PK 1+BHK 2, uncloned; SA 13/68, BHK 5, uncloned; RHO 1/48 (attenuated strain), infant mouse 28+adult mouse 89+PK 15+BHK 6, cloned; KENYA 3/57, PK 94+BHK 6, cloned; SWA 1/68, BHK 5, uncloned.
- **SAT 3 strains:** RV 7/34, PK 11+BHK 10, cloned; BEC 1/65, BHK 5, uncloned.
- **Asia 1 strains:** ISRAEL 3/63, BHK 10, cloned; PAK 1/54, BHK 3+PK 4+BHK 1, uncloned.

**Other viruses.** Reovirus, type 3, was obtained from the Central Public Health Laboratory, Colindale, as the 15th monkey kidney passage of the DEARING strain. This material was passaged subsequently in mouse fibroblast L cells and cloned by five successive isolations from single plaques. The material used for electrophoresis had received two additional passages in PK cells.

Coxsackie B6 was obtained from the Central Public Health Laboratory, Colindale, as the third monkey kidney passage of the SCHMITT strain. This material was subsequently maintained in PK cells and cloned by three successive isolations from single plaques.

Semliki Forest virus (KUMBA strain) was obtained from Dr G. N. Mowat of this Institute as the 18th BHK passage of mouse brain material and had been cloned by three successive plaque isolations.
Electrophoretic variation of FMDV

Infectivity assay. Infectivity was assayed by plaque counting on BHK cell monolayers in the case of all strains of FMDV and Semliki Forest virus. The overlay medium contained either Noble agar (Difco) or agarose, according to the plaque characteristics of the strains concerned. Reovirus infectivity was assayed on L cell monolayers by a serum-free overlay technique (Pringle & Cartwright, 1969) and Coxsackie B6 infectivity on PK monolayers.

Column zone electrophoresis. An apparatus similar to that described by Thorne (1963, Thorne et al. 1965) was constructed in the Institute workshop under the supervision of Mr H. M. Smith. A bank of six 55 x 1 cm. uniform bore electrophoresis tubes is contained in a Perspex water jacket, through which mains water can be circulated during electrophoresis. The electrophoresis tubes pass through bored-out 3/8 B.S.P. parallel stud couplings and are held in position by captive seal type nuts (Drallium Tube Couplings Ltd, Bexhill-on-Sea). Each tube is linked to an upper common electrode bath (the cathode) through detachable Perspex buffer reservoirs and tubes sealed with membranes of ‘Cellophane’. At their lower ends the tubes are connected through glass T-junctions to a common electrode bath (the anode). The T-junctions are sealed at their lower ends with ‘Cellophane’ membranes held in position by compression rings and Sira adhesive wax (B.D.H. Ltd, Poole). The side arm is connected via ‘Tygon’ tubing to a multichannel peristaltic pump and a gradient mixing device. In operation, the T-junctions are filled to the level of the side arm with 20 % (w/v) sucrose and a 50 ml. linear gradient of 5 to 20 % (w/v) sucrose is pumped slowly in parallel into the tubes. Two ml. of 2.5 % (w/v) sucrose and 2 ml. of buffer are then layered on top to form a steep 0 to 5 % gradient. The virus sample, previously dialysed to 3.6 % (w/v) sucrose and containing 0.05 % phenol red to make it visible, is introduced from above using an ‘Agla’ micrometer syringe (Burroughs Wellcome & Co., London). The optimal conditions for the electrophoresis of FMDV by this technique were established empirically. The buffer solution used in both the electrode vessels and the columns was 0.025 M-tris + HCl, pH 8.8.

A period of 1 hr was allowed for the virus zone to become stabilized before the current was applied. Under these conditions a constant voltage of 385 V gives an initial current reading of 1.5 mA per column at 10°. Electrophoresis was continued for 16 to 17 hr, by which time the phenol red marker had traversed the length of the column. Owing to the low ionic strength of the buffer, the current reading declined towards the end of the run. The reproducibility of the results was not affected, however, provided that the other conditions were held constant. When electrophoresis had been completed, the top of the column was removed down to the origin and the remainder fractionated from the bottom into approximately thirty 1.5 ml. vols by drop counting.

The electrophoresis tubes were silicone coated and 0.01 % bovine plasma albumin was included in the column buffer to improve viral recovery. The mean recovery from a random sample of 25 of the 243 gradients in this series was 58.3 %.

Virus concentration and purification. FMDV samples were concentrated approximately 100-fold by precipitation from half-saturated ammonium sulphate, resuspension and centrifugation for 1 hr at 30,000 rev./min, in the 10 x 10 ml. angle rotor of an MSE Superspeed 50 TC centrifuge. The pelleted virus was resuspended in 1 ml. 0.05 M-tris + HCl buffer, pH 7.6, and purified by the method of Brown & Cartwright (1963). Two ml. of a concentrated preparation containing 0.5 % sodium dodecyl sulphate were layered on top of an 18 ml. gradient of 15 to 45 % (w/v) sucrose in
0.05M-tris + HCl, pH 7.6, and centrifuged at 25,000 rev./min. in the 3 × 20 ml. swing-out rotor of an MSE Superspeed 50 TC centrifuge for 2½ hr. Eighteen 1 ml. fractions were collected and the virus zone located by infectivity titration.

**Complement-fixation test.** Complement-fixation activity was measured quantitatively according to the method described by Brooksby (1952) in which antigen is allowed to react with excess antiserum in the presence of increasing amounts of complement.

**Neutralization test.** Tenfold dilutions of virus samples were mixed with an equal volume of a 1/20 dilution of the appropriate hyperimmune guinea-pig serum. The mixtures were held overnight at 4°, and terminal infectivity measured by titration on BHK monolayers.

![Fig. 1. Comparison of the electrophoretic properties of an unpurified and a purified preparation of strain RHO 1/48. O—O, Unpurified sample; •—•, purified sample. The horizontal arrow indicates the movement of the phenol red marker from the origin.](image)

**RESULTS**

**Comparison of the electrophoretic properties of purified and crude virus suspensions**

Virus of strain RHO 1/48 was concentrated and purified. The fraction from the sucrose gradient with maximum infectivity was adjusted to contain 3.6% sucrose by step-wise dialysis. The purified sample and the original material were run simultaneously in parallel electrophoresis columns (Fig. 1). A single peak of infectivity was
found in each case and these were coincident; purification resulted in an improvement in resolution. The recovery of purified virus from the column, however, was poor. Adequate resolution could be obtained with unpurified material and the scope of the technique for genetic applications was greatly extended.

Effect of host cells on electrophoretic properties of the virus

Single-step mutants of strain RHO 1/48 (see Table 1) which could be differentially assayed in the same column were employed to determine whether the electrophoretic properties of unpurified virus were affected by the host cell of origin. A guanidine-sensitive mutant was passaged five times in PK cells and a resistant mutant similarly in BHK cells. The two passage materials were mixed and submitted to electrophoresis on the same column. The fractionated column was then assayed under normal and guanidine-containing overlay. The location of the infectivity peak was unaffected by short-term passage in different cells (Fig. 2).

Strain IRAQ 24/64 forms plaques on monolayers without prior adaptation to cultured cells; consequently it was possible to compare virus from bovine vesicular lesions with virus maintained in cultured cells. No electrophoretic difference was observed between vesicular material and 10th PK passage material run simultaneously in parallel columns.
The behaviour of the 7 nm. viral component of FMDV in sucrose-gradient-zone electrophoresis

The quantitative complement-fixation test was used to study the behaviour of the 7 nm. component of the protein coat of FMDV obtained by heat inactivation of the virus (Bachrach, 1961). With virus concentrated by ammonium sulphate precipitation,
the complement-fixing activity of the 7 nm. component obtained by heat treatment could not be resolved from that of untreated material (Fig. 3); both peaks ran at the same rate as infectivity.

With purified virus, however, a separation of the 7 nm. component from the virion was achieved. The complement-fixing activity and infectivity profiles of purified virus (Fig. 4a) were compared with the complement-fixing activity profiles of a 7 nm. preparation obtained by heat treatment of purified virus and an infectivity marker included at a concentration below the threshold of the complement-fixation test (Fig. 4b). The results indicated that, although the 7 nm. component had a different mobility from that of the virion, it migrated at the same rate as the virion in the unpurified state. Hence it was assumed that the differences in mobility observed between unpurified samples of strains result from differences in the subunits themselves, rather than from loss or addition of subunits.

**The resolving power of the technique**

Accurate comparison of electrophoretic mobility requires that both components should be run in the same column since minor differences in conductivity and viscosity due to variations in gradient linearity can introduce errors. In fact, with two strains with differential marker characteristics, differences in mobility could be resolved down to at least one-tenth the length of the migration path (Fig. 5). The migration path is defined as the distance moved by the phenol red marker from the origin. Calculation of the results in terms of the migration path was found to be a reliable procedure for comparing results from different columns, e.g. six separate runs of strain OVI gave values of 0.46, 0.44, 0.46, 0.44, 0.48 and 0.44.

**Comparative electrophoretic properties of different strains of FMDV**

The relative mobilities of 26 distinct strains of the seven immunological types of FMDV have been determined (Fig. 6). The strains are arranged in order of increasing mobility, relative to phenol red. The values are the means of at least two separate runs.

The range of mobilities exhibited by the FMDV strains was greater than that of the three other RNA viruses which were chosen at random. This suggests that the conditions adopted were near optimal for the differentiation of FMDV. The FMDV strains, reovirus and Coxsackie virus all gave well-defined infectivity peaks. Semliki Forest virus, on the other hand, gave a much broader profile.

The strains of FMDV all originated from field isolates obtained from different places at different times. Their subsequent in vitro histories have differed and in many cases they have distinct biological properties, e.g. strains RHO 1/48 and RV 11/37 are mouse-propagated attenuated strains. No relationship was evident between their respective biological characteristics and their electrophoretic properties. A relationship was evident, however, between electrophoretic mobility and the immunological type of the virus. There was no precise correspondence with immunological specificity since there was some overlap and two of the ten type O strains examined—O Fareham and Kenya 102/60—occupied a discrepant position. Furthermore, as will be described later, fast mutants of strains Israel 3/63 and OVI have been isolated, which were unchanged in serological specificity. In addition, within any immunological type, strains of the same sub-type differ from one another as much as strains of different sub-type. Nevertheless the observed relationship could hardly have been fortuitous.
The electrophoretic properties of some of these strains were also unrelated to certain \textit{in vitro} marker characteristics (Table I). This was particularly interesting in the case of strain \textit{KENYA 3/57}, because the large- and small-plaque variants of this strain certainly involved a surface property of the virion since these variants could be physically separated by countercurrent distribution (Pringle & Slade, 1968). Neither these variants nor the plaque morphology variants of strain \textit{ISRAEL 3/63} differed electrophoretically, whereas

![Diagram](image)

Fig. 6. A diagrammatic representation of the relative electrophoretic mobilities of 26 strains of FMDV and 3 other RNA viruses. Solid arrows represent mean mobilities determined by two methods—relative to phenol red, and relative to another viral marker in the same column. Broken arrows are mobilities determined relative to phenol red only. * The mean distance moved by the phenol red marker under these conditions was 47.5 cm.

Thorne \textit{et al.} (1965) and Breeze & Thorne (1966) were able to separate plaque morphology variants of polyoma and encephalomyocarditis virus, using the same technique of electrophoresis.

The observed electrophoretic mobilities were an intrinsic property of the virion and not the result of an association with different cellular components. This was confirmed
Electrophoretic variation of FMDV

Fig. 7. The electrophoretic separation of a purified preparation of strain KENYA 3/57 and purified ISRAEL 3/63 in the same column, titrated by differential neutralization by specific antiserum. • • •, Infectivity in presence of anti-ISRAEL 3/63 serum; ○ ○ ○, infectivity in presence of anti-KENYA 3/57 serum.

Table 1. Comparative electrophoretic mobility of various intra-strain variants of FMDV

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>Variant</th>
<th>Mobility relative to phenol red</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISRAEL 3/63</td>
<td>Asia 1</td>
<td>Large-plaque</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small-plaque</td>
<td>0.13</td>
</tr>
<tr>
<td>RHO 1/48</td>
<td>SAT 2</td>
<td>Guanidine-resistant</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guanidine-sensitive</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heat-resistant</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heat-sensitive</td>
<td>0.68</td>
</tr>
<tr>
<td>KENYA 3/57</td>
<td>SAT 2</td>
<td>Various conditional lethal mutants</td>
<td>0.66-0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large-plaque</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small-plaque</td>
<td>0.68</td>
</tr>
</tbody>
</table>
Isolation of electrophoretic mutants by serial selection

Mutants of atypical electrophoretic mobility were either absent or present at low frequency, since with three separate strains direct isolation of mutants from fractions ahead of or behind the peak fraction was not possible, even following secondary electrophoresis of these fractions. Strain ISRAEL 3/63 was chosen as the most favourable material for a sequential selection experiment to see whether, in fact, mutants of different electrophoretic properties could be isolated. A cloned isolate was submitted to electrophoresis and a fraction travelling ahead of the peak fraction was repassaged to obtain progeny virus for a second cycle of electrophoresis. This procedure was repeated three times before a response to selection was obtained (Fig. 8a to d). In a parallel experiment

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**Fig. 8.** Isolation of an electrophoretic mutant of strain ISRAEL 3/63 by 3 serial selections.  
*a to d*, Normal virus; *e to h*, 5-Fu-labelled virus.
Electrophoretic variation of FMDV

(Fig. 8e to h) using virus which had been propagated in the presence of 1 mM-5-fluorouracil (5-Fu), a known potent mutagen for FMDV (Pringle, 1968), a response to selection was obtained earlier. A mutant clone isolated by serial selection was compared with the original parental clone and the parental clone submitted to the same number of passages required to isolate the mutant (Fig. 9). The mutant clones of strain ISRAEL 3/63 were genetically stable in the sense that no reversion was evident following three cycles of multiplication with the elimination of parental virus at each stage. A reconstruction experiment showed that the fast mutant of strain ISRAEL 3/63 was at a selective disadvantage in competition with the non-mutant, although normal yields were obtained from the fast mutant propagated alone. Parental and progeny virus from a mixed infection were compared (Fig. 10). The frequency of the mutant was greatly reduced by a single passage in competition with the non-mutant and further decreased in the course of two subsequent passages.

The fast mutant of strain ISRAEL 3/63 was unaltered in serological specificity. This was shown conclusively because this strain was atypical in giving an asymmetrical reaction with anti-BEC 1/65 serum (Table 2).

Five clones isolated from the slow progeny virus peak (Fig. 10) all exhibited the
Table 2. Depression of infectivity of a strain of FMDV in presence of hyperimmune guinea-pig serum

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Clone</th>
<th>Anti-ISRAEL 3/63 serum</th>
<th>Anti-BEC 1/65 serum</th>
<th>Normal guinea-pig serum control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISRAEL 3/63</td>
<td>Parent</td>
<td>2·27*</td>
<td>1·39</td>
<td>0·10</td>
</tr>
<tr>
<td></td>
<td>Electrophoretic mutant</td>
<td>2·34</td>
<td>1·29</td>
<td>0·09</td>
</tr>
<tr>
<td>BEC 1/65</td>
<td></td>
<td>0·14</td>
<td>2·10</td>
<td>0·03</td>
</tr>
</tbody>
</table>

* Log. depression in infectivity in the presence of 1/20 hyperimmune serum.

non-mutant phenotype, i.e. the almost unimodal distribution of electrophoretic mobility of the progeny virus was not a result of extensive phenotypic mixing. In confirmation of previous findings (Pringle & Slade, 1968), it could be shown by this method
that the frequency of phenotypically mixed particles in the progeny of mixed infections with different strains could not be more than approximately $10^{-2}$. The electrophoretic phenotypes of parental and progeny virus from cells infected at high multiplicity ($\sim 10$ p.f.u./cell) with a guanidine-resistant and a guanidine-sensitive strain were determined (Fig. 11). Since intrastain recombination could be neglected, the guanidine marker located the distribution of the resistant genome. If phenotypic mixing had occurred, a bimodal distribution of the guanidine-resistant genome would be expected. The absence of phenotypic mixing has important implications in the biology of FMDV.

It was also shown, using guanidine-resistant and sensitive mutants run together on the same column, that the progeny of a clone replicated in the presence of a mutagenic concentration of 2 mM-5-Fu did not differ in electrophoretic properties from normal progeny. This observation supports the view that 5-Fu affects only the transcription and not the translation of genetic information (Heidelberger, 1965), since Munyon & Salzman (1962) have shown that 36% of the uracil in poliovirus RNA was replaced by 5-Fu in the presence of a similar concentration of 5-Fu.

A phenotypically unstable fast mutant of strain OVI

A fast mutant of strain OVI was isolated which differed from the type of mutant described previously. This mutant exhibited variable multimodal electrophoretic profiles. Repeated cloning failed to resolve it into components. Purification, detergent treatment or simply one cycle of freeze thawing, however, abolished the multimodal profile and restored the typical unimodal pattern of strain OVI. The variable phenotype of this mutant, which was a genetically stable characteristic, must be due to association of the virion with some cellular constituent. Neither the parental strain nor several others examined in this light showed evidence of phenotypic instability.

DISCUSSION

The electrophoretic properties of strains of FMDV are heritable characteristics and an intrinsic property of the virion in all cases except that of the OVI mutant described above. The cellular environment in which replication took place had no immediate effect on mobility. There was no apparent covariation between electrophoretic mobility and such biological marker characteristics as virulence, in vitro adaptation to different host cells, plaque morphology and growth characteristics. On the other hand, there was a relationship between electrophoretic mobility and immunological type, unconnected with serological specificity. The data of Hirtz (1955) and Matheka & Geiss (1965b) are in agreement with this, since in the case of the few strains studied by these authors the type O strains had higher mobilities than the type A and C strains. This relationship presumably reflects an underlying phylogenetic divergence. The reconstruction experiment with the ISRAEL 3/63 mutant indicates that the characteristic electrophoretic mobility of a strain is maintained by genetic selection at the cellular level.

The lack of an exact correspondence with specificity was not surprising, since study of the inactivating action of trypsin had indicated that only a limited portion of the protein coat of FMDV was concerned with immunogenicity (Wild & Brown, 1967). It remains to be seen whether mutants affecting immunological specificity can be isolated by this technique.
There is a parallel between the electrophoretic data and the geographical origin of the strains, suggesting that ecological factors, such as the predominant reservoir host, may be responsible for the mobility spectrum. The African strains (types SAT 1, SAT 2 and SAT 3) are ‘fast’ strains, the Asian strains (type Asia 1) ‘slow’, and the European—secondarily cosmopolitan—strains (types A, C and O) are ‘intermediate’. It may be significant that one of the two discrepant type O strains is a Kenyan strain. The origin of the other discrepant type O strain, which caused the isolated outbreak at Fareham in 1967, is unknown.

Although phenotypically mixed particles were not detected among the progeny of electrophoretically distinct strains multiplying in the same cells, this phenomenon cannot be definitely excluded. For instance, Ahl (1967) has described a case of asymmetrical marker rescue in FMDV. Nevertheless, the low frequency of phenotypic mixing means that any mutation of the genome affecting a surface property of the virion will be immediately expressed. In the case of mutations affecting antigenic properties, these would be immediately subject to selection in a natural environment consisting of populations of immune, partially immune and non-immune animals. This aspect of the biology of FMDV may be responsible in part for the extensive and apparently evolving antigenic variation of this virus. Picornaviruses which exhibit extensive phenotypic mixing, i.e. poliovirus, are more stable at least in terms of antigenicity, presumably because mutations affecting surface properties are masked and not exposed to selection in vivo. Such viruses do not exhibit the same degree of antigenic evolution as FMDV.

Poison & Deeks (1962) have observed a broad range of electrophoretic mobilities in the Coxsackie group, which might be considered as the closest relative of FMDV among the picornaviruses (Platt, 1956; Pringle, 1964). Unfortunately, Coxsackie B6 was not included in their study and a direct comparison between the two sets of data is not possible.

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Electrophoretic variation of FMDV


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