STABILITY OF FOOT-AND-MOUTH DISEASE VIRUS, ITS GENOME AND PROTEINS AT 37°C

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Summary. – Infectivity titers of foot-and-mouth disease virus (FMDV) types Asia 1 and 0 were reduced by 4 and 2 log units, respectively, after incubation at 37°C for 12 hrs. The stability of the FMDV RNA genome at 37°C was studied using ^{32}P-labelled virus. The RNA of FMDV type 0 was found to be more stable than that of type Asia 1. Oligo(dT)-cellulose chromatography showed that 21% and 31% of the labelled RNA were bound to the column in the case of types Asia 1 and 0, respectively. Possible correlation between the poly(A) tail length, accessibility of the genome to nucleases and thermostability of the infective virus is discussed. A positive correlation between the thermostability of the genome and general distribution of a particular virus type seems to exist. A stable genome associated with poor virus immunogenicity may be responsible for the prevalence of FMDV type 0 in the nature. The isoeletric focussing of structural proteins isolated from the virus samples incubated at 37°C revealed charge differences in the major immunogen between the two FMDV types. A rapid proteolytic degradation of the viral immunogen and stability of the genome may be responsible for frequent outbreaks of FMD, at least, in the endemic countries.

Key words: foot-and-mouth disease virus; genomic RNA; thermostability; poly(A) length

Introduction

FMDV belongs to the family of *Picornaviridae* containing single-stranded positive-sense RNA of size above 8 kb (Sanger, 1979). The genomic RNA of FMDV, like other picornaviruses contains poly(A) segment of 60-80 nucleotides at its 3'-end (Chatterjee *et al.*, 1976; Newman and Brown, 1976). The function of the poly(A) tail of most eukaryotic mRNAs is still obscure (Baxt *et al.*, 1979). It has been proposed that it may be necessary for efficient initiation of translation (Huez *et al.*, 1974; Doel and Carey, 1976; Hruby, 1978) or for protection against cellular nucleases (Marbaix *et al.*, 1975). The 5'-end of FMDV genomic RNA does not contain the characteristic "cap" structure but is blocked by a covalently linked viral genomic protein (VPg). This protein can be removed from the RNA by the action of a host enzyme and plays no role in the translation of the RNA (Ambros *et al.*, 1978).

It has been shown that incubation of FMDV at 37°C leads to inactivation of the virus infectivity with conservation of its physical and immunological properties. The loss in infectivity titer upon incubation of the virus at elevated temperatures was correlated with a simultaneous degradation of the 35S genomic RNA into fragments of smaller size (Brown and Wild, 1966). The ribonuclease activities have been shown to be associated internally and externally with purified FMDV. While the outer activity was easily removable, the inner one was not, and it was postulated that the enzyme was responsible for the heterogeneity normally observed in the extracted FMDV RNA (Denoya *et al.*, 1978). Reduction in the 146 S virus particles content during prolonged infection in tissue culture was reported by Suryanarayana *et al.* (1985). Nuclease associated with purified virus have also been reported in Rous sarcoma virus, vaccinia virus (Perry *et al.*, 1972) and adenovirus (Burnlingham *et al.*, 1971). The virus-associated endonuclease activity activated by NH_4^+, Ca^{++} or Mg^{++} ions was used for inactivation of FMDV vaccine strains in vaccine preparation

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Abbreviations: FMD = foot-and-mouth disease; FMDV = FMD virus; poly(A) = polyadenylic acid; VPg = viral genomic protein

(Scodeller *et al.*, 1984), and such vaccines were found to be safe and potent (Amadori *et al.*, 1987).

The 26 K viral protein (VP1) of FMDV is located on its surface. Its epitopes together with those of the other structural proteins (VP2 and VP3) induce neutralizing antibodies. The latter have been obtained also by injecting either isolated or cloned VP1 (Laporte *et al.*, 1973; Bachrach *et al.*, 1982; Pfaff *et al.*, 1982). Thus the content and stability of 146 S particles in a vaccine and the state of VP1 after storage are of prime importance for a good vaccine (Doel and Collen, 1982).

The stability of 146 S particles of type 0 strain BFS 1860 in concentrates was found to be similar to that in conventional virus preparations at 4°C but the proteolytic degradation of VP1 of 146 S particle proceeded only in concentrates and could be inhibited by the addition of ox serum. The low stability of the immunogen of virus type 0 has been recognized and it was suggested that the antigenic mass should be increased in the vaccine. It has been reported that the type 0 vaccine requires a far higher amount (220 ng) of 146 S antigen to achieve a 50% protection (PD_{50}) in cattle than do the type A (2.4 ng) and type C (4.36 ng) vaccines. It has been found that the shelf life of type 0 vaccine was about a half of that of type A or Asia 1 (Butchaiah *et al.*, 1985).

Low stability and poor immunogenicity may play an important role in the FMDV adaptation and survival in the nature. For example, the virus type 0 is widespread and found in most countries while the virus type Asia 1 is confined to the Asian countries only.

Here we report results of our studies on thermostability of infective virus particles, genomic RNA and proteins of FMDV types 0 and Asia 1, and attempt to correlate them with the occurence of these virus types in the nature.

Materials and Methods

Cells. Baby hamster kidney 21 (Glasgow, BHK-21) clone 13, maintained at the Indian Veterinary Research Institute (IVRI), Bangalore, was used for the virus propagation and infectivity titrations.

Viruses. FMDV vaccine strains type Asia 1 and type 0 maintained at IVRI, Bangalore, were used.

Infectivity titration was carried out on BHK-21 cells in microtiter plates in Eagle's Minimal Essential Medium (MEM) containing 4% foetal calf serum and 10% tryptose phosphate broth (Gibco) by a standard procedure.

^{32}P-labelling. BHK-21 cell monolayers in milk dilution bottles were infected with plaque-purified virus types Asia 1 or 0 at multiplicity of infection of 1 and incubated at 37°C for 30 mins. The monolayers were then washed four times with phosphate-free MEM devoid of serum and further incubated with the same medium containing ^{32}P-carrier free phosphate at 37°C for 12 – 16 hrs.

146 S virus particles were purified by 10 – 50% sucrose density gradient centrifugation as described by Grubman *et al.* (1979) with modifications by Surayanaryana *et al.* (1985). The virus in the peak fractions of the sucrose gradient was pelleted and resuspended in 10 mmol/l Tris HCl pH 7.4 with 100 mmol/l NaCl and 1.5 mmol/l $MgCl_2$ (reticulocyte sedimentation buffer (RSB)) and stored at -70°C.

RNA was isolated by proteinase K and phenol-chloroform extraction method as described by Grubman *et al.* (1979).

Agarose gel electrophoresis of RNA was performed on 0.6% agarose under denaturing conditions using formaldehyde as described by Lehrach *et al.* (1977). The gel after drying was subjected to autoradiography with intensifying screens at -70°C.

Poly(A) content in genomic RNA was assayed by oligo(dT)-cellulose chromatography as described by Grubman *et al.* (1979). The percentage of bound (poly(A) RNA) and unbound (poly(A)-free RNA) counts was calculated.

Southern blot analysis was done as described by Maniatis *et al.* (1982). The BHK-21 cell genomic DNA was isolated by the method of Blin and Stafford (1976).

Electrofocussing of viral structural proteins was done in polyacrylamide slab gels (10 x 0.1 cm) containing 4% ampholytes (pH 3.5 – 10). The viral proteins obtained from 50 g of purified virus (concentration calculated on the basis of formula A_{260} 7.6 = 1 mg/ml, Bachrach *et al.* (1964)) were dissolved in 15 µl of RSB, mixed with equal volume of buffer consisting of 2% SDS, 20 mmol/l Tris, 4% ß-marcaptoethanol, and 20% glycerol, and heated in boiling water for 1 min. A 20 µl aliqout was spotted on glass fibre strip placed on the gel at the cathode end. The pH gradient was generated using 0.1 mol/l NaOH at the cathode and 0.1 mol/l phosphoric acid at the anode side. The samples were focussed towards the anode.

Results

Stability of virus infectivity at 37°C

Samples of purified FMDV types 0 and Asia 1 dissolved in RSB were incubated at 37°C for 6 and 12 hrs and titrated. Samples stored at -70°C served as controls. The results are summarized in Fig. 1. The virus infectivity titer (log $TCID_{50}$/ml) of type Asia 1 at zero hour was 8.43. It dropped after 6 and 12 hrs of incubation to 6.19 and 4.19, respectively (a drop by 4 log units after 12 hrs of incubation). Type 0, on the other hand, had an initial titer of 6.43/ml which after 6 and 12 hrs fell to 5.19 and 4.19, respectively (a drop by 2 log units after 12 hrs of incubation).

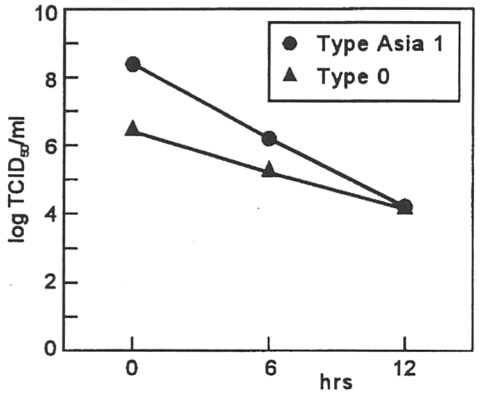

Fig. 1
Thermostability of FMDV types Asia 1 and 0 at 37°C
Virus samples incubated at 37°C for 6 and 12 hrs were titrated for $TCID_{50}$. Samples kept at -70°C served as controls (hr 0).

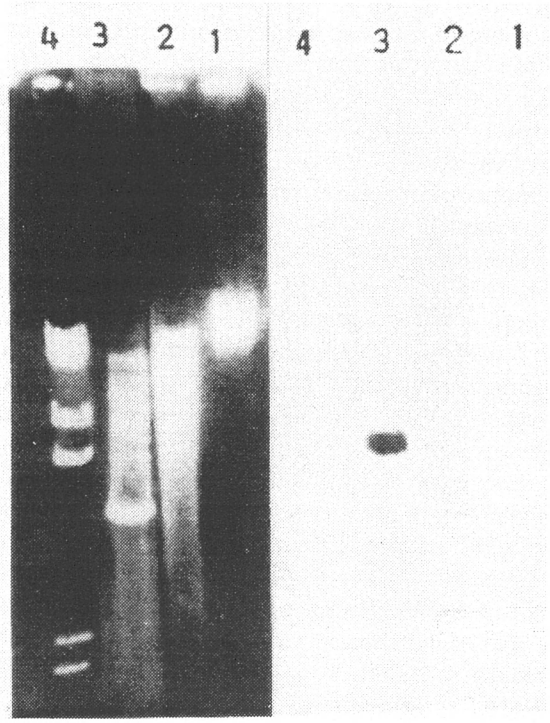

Fig. 2
Southern blot analysis of ^{32}P-labelled FMDV RNA
BHK-21 cell DNA, undigested (lane 1); *Eco*RI-digested cell DNA (lane 2); *Eco*RI-digested FMDV type Asia 1 cDNA (lane 3); size marker DNA fragments (23, 9.4, 2.3, and 2.0 kbp). Left side: ethidium bromide-stained 0.6% agarose gel after electrophoresis. Right side: the same gel after blotting, incubation with ^{32}P-labelled FMDV RNA (mixture of both types Asia 1 and 0) and autoradiography.

Nucleolytic activity on RNA

The RNA genomic RNAs of FMDV types 0 and Asia 1 were labelled *in vitro* with ^{32}P-phosphate. The purity of the RNA preparations obtained was assayed by Southern blot analysis (Fig. 2). The cloned *Eco*RI-digested Asia 1 cDNA and cellular DNAs were denatured, electrophoresed, blotted and hybridized with ^{32}P-labelled FMDV RNA. As shown in Fig. 2, the labelled RNA hybridized only with cloned Asia 1 cDNA (Fig. 2, lane 3). There was no hybridization signal with either *Eco*RI-digested or undigested BHK-21 cell DNA (Fig. 2, lanes 1 and 2). In order to study the stability of viral genomic RNA the ^{32}P-labelled virus samples were incubated at 37°C for 6 and 12 hrs while samples kept at -70°C served as controls. The RNAs were extracted from these samples and electrophorese in 0.6% agarose gel under denaturating conditions. The viral RNA reported to be 8 kb long (Sanger *et al.*, 1979) showed a marked degradation during incubation (Fig. 3, lanes 3-6). The degradation was apparently stronger in the case of type Asia 1, because there was a more marked decrease in the radioactivity (Fig. 3, lanes 3-5) during incubation for 6 and 12 hrs. On the other hand, RNA of virus type 0 showed high molecular mass species even after incubation for 6 and 12 hrs (Fig. 3, lanes 4-6). Thus RNA of virus type 0 was more stable than that of virus type Asia 1. Even though the RNAs have shown extensive degradation in the absence of RNAse inhibitors, the difference between the two types was evident. These data correspond to those obtained in studies on the stability of the infectivity titer.

Length of poly(A) tail

To detect the presence of poly(A) tails, the labelled RNAs isolated from purified virus stored at -70°C were subjected to affinity chromatography on oligo)dT)-cellulose (Table 1). The oligo(dT)-cellulose-bound fraction of type Asia 1 RNA was about 21% of the total radioactivity. On the other hand, about 31% of the total counts was bound in the case of type 0 RNA. These results indicated that the type 0 RNA had either a longer poly(A) tail or was polyadenylated to a greater extent than the type Asia 1 RNA.

Electrofocussing studies on structural proteins

Purified samples of virus types Asia 1 and 0 were incubated at 37°C for 6 and 12 hrs and subsequently the structural proteins were subjected to electrofocussing using standard markers of known pI values.

As shown in Fig. 4 (lanes 2,4) the pI value of VP1 of type Asia 1 (9.0) was by 0.2 units higher than that of type 0

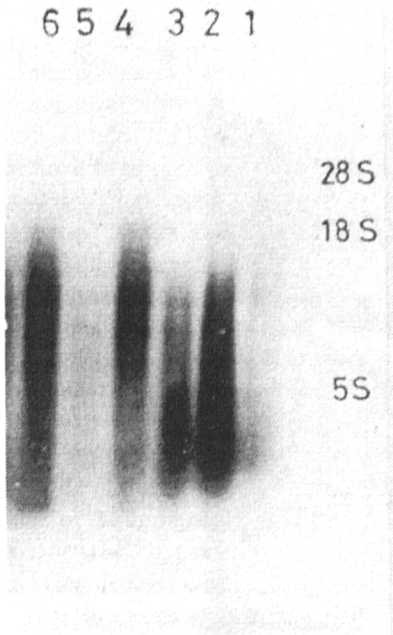

Fig. 3
Effect of incubation at 37°C on ^{32}P-labelled genomic RNA of FMDV types Asia 1 and 0

Samples of isolated ^{32}P-labelled RNAs were incubated at 37°C for 6 and 12 hrs, electrophoresed in 0.6% agarose gel in denaturing conditions, dried and autoradiographed. Non-incubated (control) RNAs of virus type Asia 1 (lane 1) and 0 (lane 2); RNAs incubated for 6 hrs, types Asia 1 (lane 3) and 0 (lane 4); RNAs incubated for 12 hrs, types Asia 1 (lane 5) and 0 (lane 6). Positions of size marker RNAs are shown on the right side.

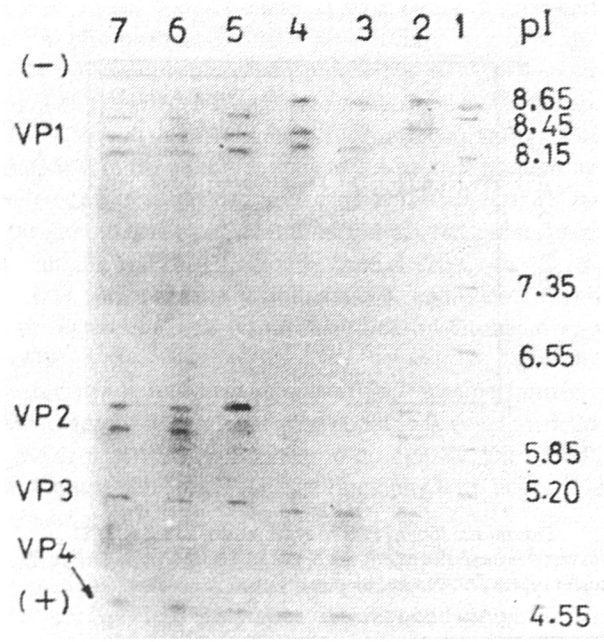

Fig. 4
Isoeletric focussing of FMDV structural proteins

Samples of purified FMDV types Asia 1 and 0 were incubated at 37°C for 6 and 12 hrs, and subjected to electrofocussing on 4% polyacrylamide gel. Samples kept at -70° served as controls (hr 0). PI markers (lane 1); type Asia 1 proteins after 0, 6 and 12 hrs (lanes 2, 3, 4); type 0 proteins after 0, 6 and 12 hrs (lanes 5, 6, 7). Positions of VP1, VP2, VP3 and VP4 are indicated on the left, and positions of pI markers are on the right.

Table 1. Oligo(dT)-cellulose chromatography of FMDV genomic RNAs of virus types Asia 1 and 0

Virus type	cpm(%)		
	Unbound	Bound	Total
Asia 1	10.548 (78.46)	2.895 (21.5)	13.443 (100)
0	13.614 (68.26)	6.330 (31.73)	19.944 (100)

(8.8) (Fig. 4, lanes 5-7). This indicates that VP1 of type Asia 1 is more basic than that of type 0.

The pI value of type Asia 1 VP2 ranged from 5.8 to 6.0 (Fig. 4, lanes 2-4), similarly to type 0 VP2 (pI 6.05) (Fig. 4, lanes 5-7).

The VP3 of types Asia 1 and 0 was found to have pI values of 5.05 and 5.1, respectively, indicating that there was no difference in the case of VP3 too. Similar results were obtained also with VP4 (Fig. 4).

There was no effect on VP1 of type Asia 1 upon incubation at 37°C for 6 and 12 hrs. However, the VP2 protein has shown an additional band at pI 4.9 with a simultaneous decrease in the intensity of the two bands seen in the control (Fig. 4, lane 2). The VP3 protein was found intact after incubation at 37°C in both cases.

The bands of structural proteins VP1 and VP2 in the case of type 0 did not show significant difference upon incubation at 37°C for 6 or 12 hrs (Fig. 4, lanes 5-7). A reduction in the number of bands of VP2 was seen also in the case of incubation at 37°C for 12 hrs (Fig. 4, lane 7).

Discussion

The temperature efffect on the stability of FMDV has been studied earlier by Bachrach (1966). The instability of FMDV RNA at 37°C due to virus-associated nucleolytic degradation was reported by Denoya *et al.* (1978). The results of our experiments on the stability of FMDV infectivity at 37°C showed that both the types Asia 1 and 0 were quite unstable at this temperature, the type Asia 1 more than the type 0. In general, the observed reduction in the virus infectivity titer might be due to a loss in virus attachment sites on the virion surface and/or to a degradation of viral genome. As the FMDV genomic RNA is infective and the

drop of infective titer observed by us was as high as 2 – 4 log units for the two virus types, a mere loss of virion attachment sites could not account for this phenomenon. It has been reported by Denoya *et al.* (1978) that the FMDV infection does not require cellular receptors. Therefore it was apparently the virus-associated nucleolytic activity which was responsible for the reduction in the virus infectivity titer observed in our experiments. The difference between the types Asia 1 and 0 may be explained by a higher nucleolytic activity in type Asia 1, and/or by a better accessibility of genomic RNA to nuclease(s) in the case of type Asia 1 caused by stripping off of virion surface protein(s). However, we have so far no information on qualitative or quantitative differences between nucleases associated with FMDV types Asia 1 and 0.

Our experiments on the stability of ^{32}P-labelled FMDV RNA at 37°C revealed a much higher nucleolytic activity associated with type Asia 1 as compared to type 0. It was demonstrated that type Asia 1 RNA after 12 hrs at 37°C was almost completely degraded to oligonucleotides or nucleotides. The RNAs were extracted in the presence of ribonucleotide-vanadyl complexes, an RNase inhibitor preventing enzymatic RNA degradation. In spite of that the isolated viral DNA was considerably degraded at 37°C or -70°C (control), though some RNA remained intact (8 kb). The type Asia 1 RNA showed after 6 hrs at 37°C some low molecular mass fragments (oligonucleotides), but the latter disappeared after 12 hrs.

The degradation of FMDV genomic RNA might be also caused by nucleolytic activity associated with the capsid proteins, though such activity was not reported so far. Eukaryotic RNAs are known to be stabilized during translation by the poly(A) tails at the 3'-end (Marbaix *et al.*, 1975). It has been reported that the 5'-end of FMDV genomic RNA is linked with the VPg protein (Ambros *et al.*, 1975). The 3'-end is known to have a poly(A) tail of variable length whose role in translation has not been elucidated so far. The residues of the poly(A) tail are presumably steadily removed by a 3'-exonuclease and simultaneously added by a poly(A) polymerase.

The percentage of poly(A)-free RNA in total RNA was estimated at 78% and 68% for FMDV types 0 and Asia 1, respectively. It seems that the 3'-end of type Asia 1 RNA was a little more susceptible to the nuclease(s) associated with virions or contaminating them.

Furthermore it can be assumed that the universal distribution of FMDV type 0 may be due to its relatively stable genome. On the other hand, FMDV type Asia 1 having a relatively unstable genome is very much restricted to Asian countries. A similar reasoning can be applied to the SAT 1 strain of FMDV. What concerns the frequent outbreaks of FMDV type 0 they may be conditioned besides by the stable genome also by its poor immunogenicity. Pay and Hingley (1987) have observed that a far higher dose (220 ng) of the 146 S antigen was needed to achieve a 50% protection in cattle with FMDV type 0 than with FMDV types A (2.4 ng) and C (4.36 ng).

To elucidate the poor immunogenicity of the structural protein VP1 or of 146 S particles of FMDV the experiments on electrofocussing of the capsid proteins were performed. They revealed that pI of VP1 of type Asia 1 was higher (9.0) than that of type 0 (8.8). The higher basicity of type Asia 1 VP1 may promote the aggregation of virus particles which in turn may help in protecting the protein against a proteolytic action. It is predominantly the type Asia 1 that yields a small plaque variant with a tendency to aggregate (Cowan *et al.*, 1974). Furthermore our experience shows that a better recovery of the type Asia 1 can be achieved by mild detergent treatment (V.V.S. Surayanarayana, personal communication). We have also observed that electrophoretic bands of VP1 were weaker in purificates of FMDV type Asia 1 stored at -70°C for 3 months. One possible explanation of this observation could be a stripping off of VP1 which could subsequently lead to faster degradation of viral genomic RNA because of its better accessibility to nucleases. Or, VP1 of this virus type could be more susceptible to virion-associated or contaminating proteases. A relatively poorer immunogenicity of FMDV type 0 (Pay and Hingley, 1987) in comparison with other types may be ascribed to a possibly lower stability of VP1 of this virus type. However, we have no experimental evidence on this subject.

In conclusion, FMDV type 0 has apparently a stable genome but is an unstable and/or poor immunogen. These properties may favour its ability to survive in the nature and cause frequent outbreaks. On the other hand, the genome of FMDV type Asia 1 is highly unstable and the immunogenic protein of this virus is highly basic. These properties may be somehow linked to its confinement to some countries only.

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