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Guanidine and heat sensitivity of foot-and-mouth disease virus (FMDV) strains

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SUMMARY

A study of the ability of 49 strains of FMD virus to replicate in BHK-21 monolayer cells maintained under a standard agar overlay containing 5·2 mM guanidine hydrochloride and to withstand heat inactivation at 54 °C for 1 h showed that strains belonging to serotypes C, O and Asia 1 were generally more resistant to guanidine and heat stable than the SAT 1, 2 and 3 serotypes. The type A viruses as a whole occupied an intermediate position between these two groups.

In vitro passage in BHK-21 cells influenced the guanidine sensitivity of 3(O, C and SAT 3) of the 7 FMD serotypes suggesting that this is not a stable genetic marker. Heat stability of the FMD viruses, however, did not change on passage, suggesting that this is a stable characteristic inherent in any homogeneous FMD virus population.

INTRODUCTION

There are seven serotypes of foot-and-mouth disease virus (FMDV). These are the European types O, A and C now found in many parts of the world, type Asia 1 found in the Middle and Far East, and the Southern African Territories types SAT 1, SAT 2 and SAT 3, which are present in many regions of Africa.

These types can be differentiated by cross-protection tests in convalescent animals or by serological tests in the laboratory. Within each serotype subtypes can be distinguished (Pereira, 1977) and the antigenic variation among these may be such that animals vaccinated against one are insufficiently protected against others. Manufacturers supplying vaccines to several countries must therefore be capable of making vaccines to protect against a wide range of subtypes as well as having the facility to respond quickly to the emergence of a new subtype.

Type O, A, C and Asia 1 viruses used to prepare vaccines in BHK-21 suspension cell cultures on an industrial scale have regularly resulted in potent stable vaccines, whereas vaccines prepared from SAT viruses have generally been less potent and less stable (Mowat, 1974; Pay & Schermbrucker, 1974; Chema & Rweyemamu,

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1978; Pay, Rweyemamu & O'Reilly, 1978). The reasons for this are not fully understood even though the SAT viruses have been reported to differ from other FMDV types in that they are more sensitive to the anti-viral compound guanidine hydrochloride (Pringle, 1964*a*), have faster electrophoretic mobilities (Pringle, 1969) and are distinct on the basis of RNA sequence homology (Robson, Harris & Brown, 1977). These studies, however, used only a relatively small number of well adapted laboratory strains and it is important to know if this distinction would be substantiated when many subtypes with different passage histories were examined.

In this investigation we have studied the ability of a large number of FMDV subtypes to replicate in the presence of guanidine hydrochloride and to withstand heat inactivation at 54 °C for 1 h. Both these properties have been recognized as heritable characteristics which have been widely used in genetic studies of FMD viruses. Sensitivity to guanidine has played a major role in the genetic mapping of FMDV (Lake, Priston & Slade, 1975; McCahon *et al.* 1977) and together with heat inactivation at 54 °C, has previously been used in the study of FMDV recombinant progeny (Pringle, 1965; Mackenzie & Slade, 1975).

MATERIALS AND METHODS

Virus Preparations and Assays

The FMD virus strains studied were from the vaccine seed bank. The original field isolates had been obtained from the World Reference Laboratory for FMD, Animal Virus Research Institute, Pirbright and passaged a number of times in primary calf thyroid cultures and BHK-21 clone 13 monolayer cultures. Some of the strains had also been passaged in BHK-21 suspension cultures (Capstick *et al.* 1962). The viruses were all harvested as extracellular fluids from BHK-21 monolayer cells maintained in Eagle's medium supplemented with 10% tryptose phosphate broth and 1% adult bovine serum. Cell debris was removed by centrifugation at 600 ***g*** for 10 min and the serotype specificity of the clarified virus was checked by the microneutralization test, which has been shown to be more sensitive than the conventional complement fixation test and capable of detecting low quantities of heterotypic viral contamination (Rweyemamu *et al.* 1978). Small volumes were stored at −70 °C. Virus was assayed in BHK-21 monolayer cells at 36 °C by the plaque titration method of Mowat & Chapman (1962), using 1% agar in tris-buffered Eagle's medium as overlay. The overlay contained 150 μg/ml of DEAE-dextran. Plaques were counted after 48 h at 36 °C. All the virus strains tested had initial infectivity titres between 6·0 and 7·5 $\log_{10}$ p.f.u./ml.

Sensitivity to Guanidine and Heat

A total of 49 FMD virus strains were tested representing all seven serotypes (Table 1).

The strains were screened for their ability to replicate in the presence of guanidine hydrochloride and to withstand heat inactivation using duplicate 1:10 dilutions of stock virus made in complete phosphate buffered saline (PBS), pH 7·6.

Unheated samples were assayed under standard overlay and overlay containing 5·2 mM guanidine hydrochloride, and virus was also assayed under standard overlay following incubation for 1 h in a waterbath at 54 °C. All viruses were tested at least twice.

RESULTS

Since the passage history of the viruses was different it was essential to determine if *in vitro* passage of the strains exerted a significant influence on their susceptibility to either guanidine or heat. As is evident from Table 1 there was no relationship between sensitivity or resistance to guanidine or heat and previous culture of virus strains in BHK suspension cells. Similarly when all the virus strains were considered together there was no significant correlation between the passage level and guanidine or heat sensitivity (Fig. 1). However, an examination of virus strains of each of the seven serotypes revealed a negative correlation between number of passages and sensitivity to guanidine for serotypes O ($r = 0{\cdot}70$) and SAT 3 ($r = 0{\cdot}995$) viruses, and a positive correlation for type C viruses ($r = 0{\cdot}81$) (Fig. 2). Four virus strains (O Pacheco, A Morocco 5/77, A Philippine 10/75 and SAT 1 Sudan 8/74) were studied at low and high passage levels (Table 1). Only the O Pacheco strain showed an alteration in its sensitivity to guanidine confirming the increasing resistance of type O viruses to guanidine on passage in cell cultures. There was no significant correlation demonstrated between the passage level and heat sensitivity whether all the 49 strains were analysed together or as individual serotypes.

The distribution of the two marker characteristics among the 49 FMDV strains was further analysed using a multiple range test to compare the weighted mean infectivity titre reductions for each treatment. Use was made of the standard deviations and the number of observations for each serotype mean. Table 2 shows the weighted means, and those groups of means which show no difference at the 5% level of significance are bracketed together. Thus growth in the presence of guanidine divided the 49 strains of the seven serotypes into three distinct groups; group I, type C viruses; group II, types O, Asia 1 and A viruses and group III, types SAT 3, SAT 2, and SAT 1 viruses which were the most sensitive.

Heat treatment also divided the FMDV serotypes into three distinct categories according to their degree of stability; group I, the stable types C, Asia 1 and O viruses; group II, types A, SAT 2 and SAT 1 viruses and group III, type SAT 3 viruses.

It was evident from the above studies that virus strains belonging to the SAT serotypes were sensitive to heat and guanidine. It was, therefore, decided to compare the kinetics of heat inactivation at 54 °C of strain SAT 1 SAR 4/74 representing heat-labile viruses and strain O_1BFS 1860 representing the relatively heat stable serotypes. The result of such an experiment is shown in Fig. 3 from which it can be seen that the inactivation slope for O_1BFS 1860 was shallow and roughly linear with a half-life of approximately 8 min. In contrast the inactivation curve of SAT 1 SAR 4/74 virus was steep and curvilinear showing an initial rapid

Table 1. *Reduction in infectivity of FMD virus strains caused by growth under overlay medium containing* 5·2 mM *guanidine hydrochloride or heat inactivation at* 54 °C *for* 1 *h* (*each virus was tested at least twice*)

Virus strain	No. of passages	Loss of titre (log_{10} p.f.u./ml) after Guanidine treatment	Heat inactivation
O_1BFS 1860	16	2·94 ± 0·82 ($n = 4$)	2·38 ± 0·69 ($n = 3$)
O Lausanne	15	1·03 ± 0·31 ($n = 3$)	2·29 ± 0·45
O Pacheco	14	0·62 ± 0·24 ($n = 4$)	1·82 ± 0·22 ($n = 3$)
O Pacheco	3	5·01 ± 0·90 ($n = 3$)	2·01 ± 0·18 ($n = 3$)
O Campos	7	3·69 ± 0·16	2·31 ± 0·01
O Colombia 7250	9	3·95 ± 1·45	3·32 ± 0·57
O Sudan 5/75	6	3·35 ± 0·89	2·52 ± 0·13
O Sudan 6/75	7	1·13 ± 0·01	2·99 ± 0·03
O Sudan 1/76	7	4·61 ± 0·64	3·32 ± 0·51
OK 120/64	16	1·03 ± 0·24	3·65 ± 0·02
A_5 France 1/68	18	1·08 ± 0·12	2·24 ± 0·23
A_{22} Mahmatli	14	4·4 ± 0·56	1·77 ± 0·25
A_{22} Iraq 24/64	14	5·39 ± 0·25	1·63 ± 0·16
A Pando (1970)	20	1·69 ± 1·38	1·58 ± 0·02
A_{24} Cruzeiro	15	1·12 ± 0·11	1·81 ± 0·18
A Colombia 8046	7	3·64 ± 0·72	3·91 ± 0·25
A Bage	11	4·14 ± 0·75 ($n = 3$)	4·02 ± 1·07 ($n = 3$)
A Venceslau (Bra 1/77)	5	3·64 ± 0·04	2·37 ± 0·16
AK 18/66	18	3·20 ± 0·14	3·27 ± 0·54
A Sudan 2/75	9	3·01 ± 0·35	3·94 ± 0·91
A Morocco 5/77	4	4·01 ± 0·32	3·23 ± 0·17
A Morocco 5/77	24	4·26 ± 0·16	3·93 ± 0·25
A Morocco 8/77	4	4·33 ± 0·49	4·86 ± 0·52
A Philippine 10/75	3	3·44 ± 0·35	4·97 ± 0·31
A Philippine 10/75	17	4·58 ± 0·45 ($n = 3$)	4·91 ± 0·16 ($n = 3$)
A Philippine 14/75	4	3·79 ± 0·58 ($n = 3$)	4·30 ± 0·95 ($n = 3$)
C Noville	19	2·18 ± 1·09	2·21 ± 0·14
C_3 Indaial	?	1·61 ± 1·0 ($n = 3$)	1·66 ± 0·08
C_3 Resende	6	1·53 ± 1·88	1·97 ± 0·24
C Pando	3	Titre increased by 0·38 ± 0·13	1·77 ± 0·15
C Philippine 7/76	10	0·94 ± 0·35	1·76 ± 0·13
Asia 1 Pak 1/54	12	0·18 ± 0·25	2·19 ± 0·01
Asia 1 Iran 1/73	8	3·04 ± 1·22	2·24 ± 0·02
Asia 1 Turkey 73	13	4·58 ± 0·60	2·55 ± 0·35 ($n = 3$)
Asia 1 Hong Kong 24/75	3	2·80 ± 0·30	2·49 ± 0·35
SAT 1 Bot 1/68	14	> 4·49 ± 0·01	3·10 ± 0·27
SAT 1 SAR 1/71	2	5·17 ± 0·18	4·35 ± 0·78
SAT 1 Sudan 8/74	8	4·81 ± 0·16	3·64 ± 0·18
SAT 1 Sudan 8/74	15	4·80 ± 0·44	3·54 ± 0·30
SAT 1 T 155/71	7	4·71 ± 0·01	4·73 ± 0·04
SAT 2 Bot 3/77	6	> 4·36 ± 0·14 ($n = 3$)	3·81 ± 0·61
SAT 2 Ken 3/57	13	4·24 ± 0·08	5·01 ± 1·0
SAT 2 K 227/66	6	4·87 ± 0·54	3·38 ± 0·4
SAT 2 K 183/74	16	5·31 ± 0·06	4·18 ± 1·17

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Table 1 (*cont.*)

Virus strain	No. of passages	Loss of titre (log_{10} p.f.u./ml) after Guanidine treatment	Heat inactivation
SAT 2 Moz 1/70	11	$> 4{\cdot}58 \pm 0{\cdot}67$	$3{\cdot}91 \pm 0{\cdot}28$
SAT 2 Rho 2/72	4	$> 4{\cdot}28 \pm 0{\cdot}39$ ($n = 3$)	$2{\cdot}45 \pm 0{\cdot}41$
SAT 2 SWA 1/68	10	$> 5{\cdot}39 \pm 0{\cdot}83$	$3{\cdot}86 \pm 0{\cdot}72$
SAT 2 Swz 1/69	21	$> 4{\cdot}69 \pm 0{\cdot}01$	$3{\cdot}52 \pm 0{\cdot}59$
SAT 2 Tan 5/68	8	$> 5{\cdot}30 \pm 0{\cdot}16$	$> 5{\cdot}43 \pm 0{\cdot}02$
SAT 2 Uga 6/70	15	$> 5{\cdot}04 \pm 0{\cdot}13$	$3{\cdot}15 \pm 0{\cdot}08$
SAT 3 Bec 1/65	34	$4{\cdot}05 \pm 0{\cdot}11$ ($n = 4$)	$4{\cdot}84 \pm 0{\cdot}22$ ($n = 5$)
SAT 3 Malawi 3/76	5	$> 5{\cdot}55 \pm 0{\cdot}30$	$3{\cdot}75 \pm 0{\cdot}74$
SAT 3 Rho 1/74	10	$5{\cdot}15 \pm 0{\cdot}58$	$4{\cdot}76 \pm 0{\cdot}76$

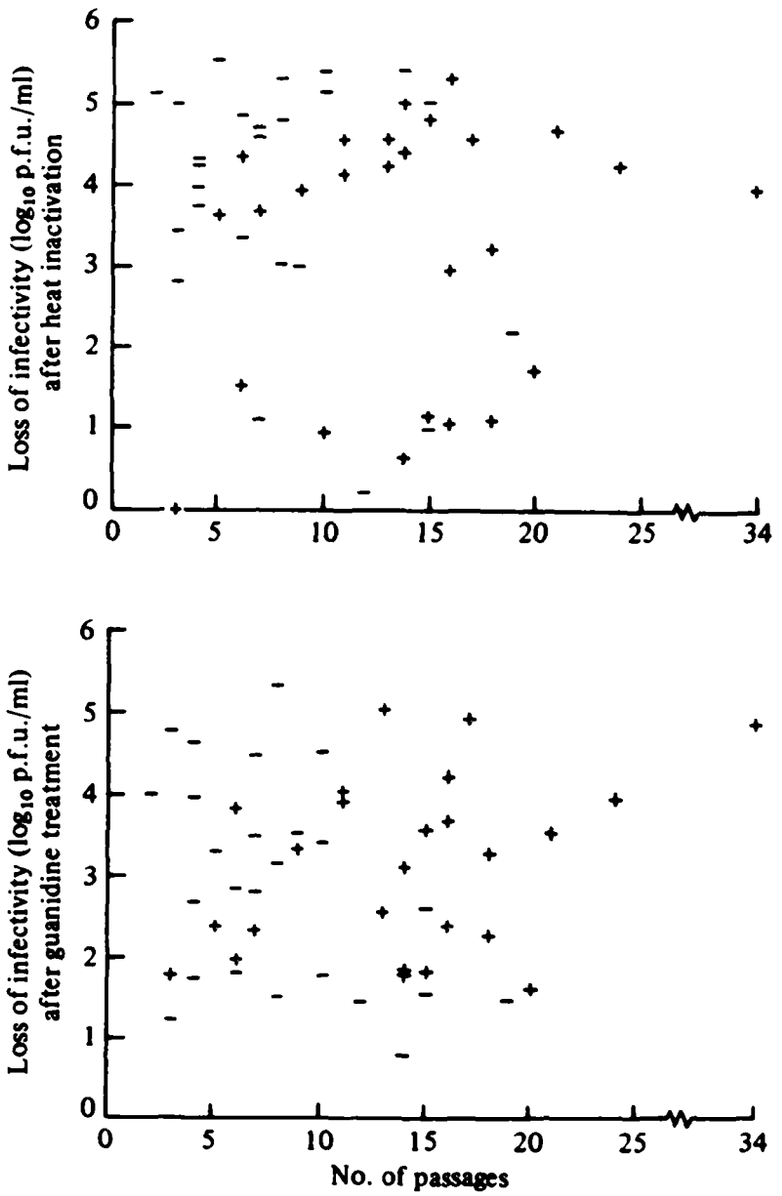

Fig. 1. The effect of number of passages in laboratory cells on the guanidine and heat sensitivity of FMDV subtypes. Subtypes adapted to growth in BHK 21 suspension cells are shown by +. Those grown only in BHK 21 monolayer cells are shown by −.

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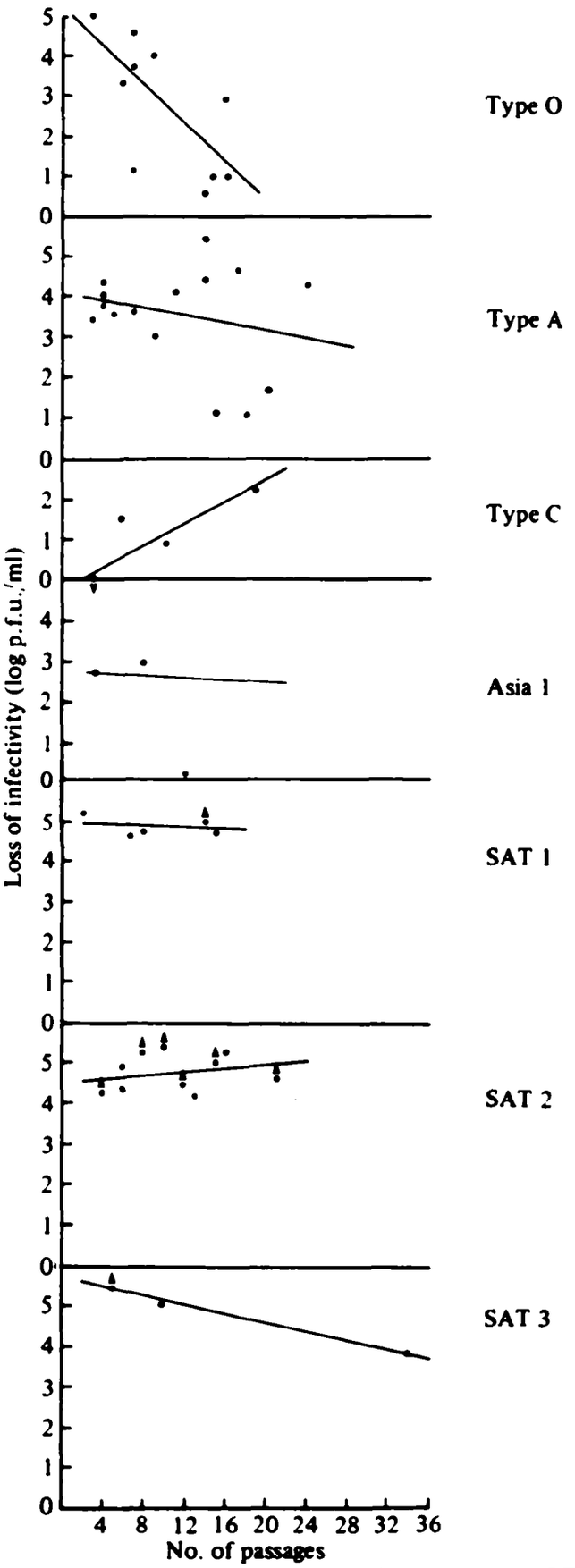

Fig. 2. The relationship between *in vitro* passage and sensitivity of FMD virus strains to guanidine hydrochloride. Data for each virus serotype were pooled and each dot represents mean reduction in infectivity from two or more replicates at each virus passage level. Where exact reduction was not obtainable the dots are arrowed to show less than or greater than the indicated level.

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Table 2. *The weighted mean reductions in infectivity titre due to guanidine overlay and heat inactivation treatments*

Guanidine overlay treatment		Heat treatment	
Virus strain	Weighted mean	Virus strain	Weighted mean
C	1·18	C	1·85
O	2·75	Asia 1	2·37
Asia 1	2·82	O	2·53
A	3·54	A	3·39
SAT 3	4·27	SAT 2	3·80
SAT 2	4·81	SAT 1	3·87
SAT 1	4·90	SAT 3	4·55

The braces show those groups of means for which no significant differences were found in a multiple range test (at the 5% level).

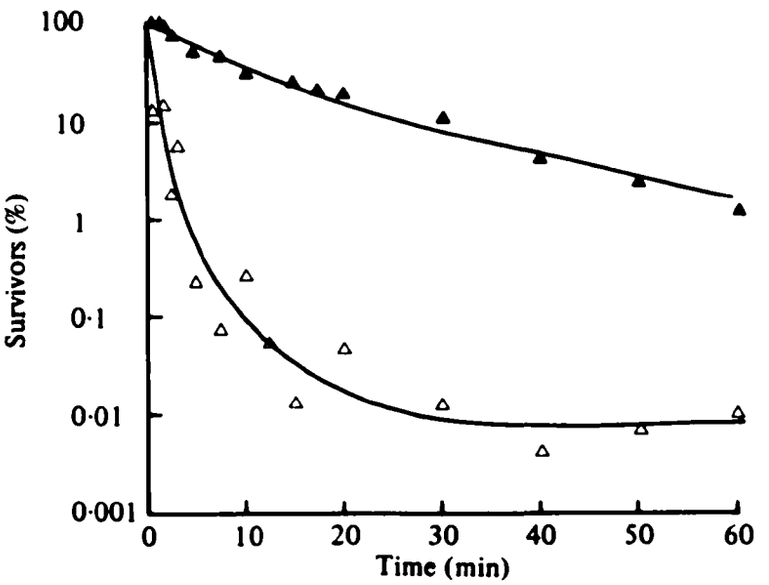

Fig. 3. Inactivation curves at 54 °C for two FMD viruses, ▲, O_1BFS 1860; △, SAT 1 SAR 4/74.

loss of infectivity in which the half-life was less than a minute followed by the persistence of a low level of infective virus. In order to assess whether such a persistent fraction represented a proportion of virus which was truly heat resistant, an attempt was made to isolate heat stable populations from the surviving fraction of strains SAT 1 SAR 4/74 and SAT 3 Bec 1/65 after heating at 54 °C for 60 min. At the end of each inactivation period the surviving virus was propagated in BHK cells to high titre and progeny virus further tested for heat resistance. Even after three such passages there was no significant difference in the inactivation kinetics of viruses passaged with or without heat selection. The apparently heat resistant fraction was probably an artefact induced by the presence of some stabilizing factor in the sample. Further evidence for this was provided by an experiment in which SAT 1 SAR 4/74 virus with an initial titre of 7·7 $\log_{10}$p.f.u./ml was heated at 54 °C for 1 h; a sample was then titrated and another aliquot was diluted 1:2 in fresh

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buffer and reincubated at 54 °C together with the undiluted original sample. The titre of the original sample was 4·0 $\log_{10}$p.f.u./ml at the end of the initial 1 h incubation at 54 °C. In contrast, the diluted sample had lost infectivity completely by 20 min of re-incubation.

DISCUSSION

The almost total inability of all the 17 strains of SAT serotypes to replicate in the presence of guanidine was a striking feature of our studies. This observation amplifies the report by Pringle (1964*a*) who showed that, of eight strains tested, the four SAT strains were among those most severely inhibited by guanidine.

Within the O, Asia 1 and A serotypes studied the degree of inhibition covered a wide range, although there was a trend for the A viruses as a whole to grow less well in the presence of guanidine. In contrast to the report by Brown (1976) only the type C viruses were significantly unaffected, and one of these, C Pando, was totally uninhibited by the concentration of guanidine used. The type C viruses were also unusual in that *in vitro* passage increased their sensitivity to guanidine. Conversely type O and type SAT 3 showed a decrease in sensitivity on passage, and thus it would appear that BHK-21 cell mediated selection can change the guanidine sensitivity of an original virus population, providing evidence that, for these three serotypes at least, it is not a stable genetic marker.

How guanidine inhibits the growth of FMDV is not yet fully understood. Guanidine has been shown to interfere with the formation of viral RNA polymerase (Brown, Martin & Underwood, 1966), to inhibit viral replication later in the growth cycle (Black & Brown, 1969), and the effect of guanidine on certain A, O and C strains has also been shown to be strongly enhanced at supraoptimal temperatures (Nick & Ahl, 1976). Further investigations into the obviously complex mode of action of guanidine are needed, and it is clear from our results that among FMD viruses, strains can be selected which will show any required degree of susceptibility.

Heating FMD viruses at 54–56 °C results in a loss of infectivity and this has been associated with a combination of RNA degradation and the disruption of the nucleocapsid (Bachrach, 1964; Brown & Crick, 1958; Rweyemamu, Terry & Pay, 1979) unlike inactivation at 37 °C which is predominantly a function of *in situ* RNA degradation (Brown & Wild, 1966). Since the immunogenicity of the virus is dependent primarily on the integrity of the nucleocapsid (Cowan, 1973), the heat stability marker could be expected to give an indication of the likely stability of the capsid during vaccine manufacture and storage. There appears to be a correlation between heat stability and immunogenicity for the majority of viruses studied since the stable types C, Asia 1 and O viruses result in potent vaccines that are stable for periods in excess of a year at refrigeration temperature (Mowat, 1974) while vaccines from most SAT viruses are less potent and less stable (Pay & Schermbrucker, 1974; Chema & Rweyemamu, 1978; Pay, Rweyemamu & O'Reilly, 1978). The type A viruses as a whole were as heat labile as the SAT viruses, but this result was probably influenced by the instability of the new isolates from

Sudan, Philippines and Morocco, whose immunogenicities have yet to be fully evaluated and strains Colombia 8046 and Bage which have now been abandoned on account of their inconsistent performance as vaccine strains. However, the established highly immunogenic type A vaccine viruses, A_{24} Cruzeiro, A Pando (1970), A_{22} Mahmatli and A_5 France 1/68 were all clearly heat stable. Recently, Doel & Baccarini (1981) studied the thermal (49 °C) stability of 140S particles from one representative strain of each of the seven serotypes of FMDV and also found the SAT viruses to form a group of relatively unstable viruses.

The finding that SAT serotypes were guanidine and heat sensitive may be linked to a more fundamental genetic characteristic. In contrast to the reports of Bachrach, Patty & Pledger (1960) and Pringle (1964*b*) we were unable to select heat stable mutants from the SAT viruses suggesting the viruses to be homogeneous in this respect. Further evidence for the uniqueness of SAT serotypes comes from the RNA hybridization studies of Robson *et al.* (1977) who found a high degree of homology among serotypes O, A, C and Asia 1 and a similarly high homology among serotypes SAT 1, SAT 2 and SAT 3 but very low homology between the two groups. It is becoming increasingly evident that the SAT viruses differ considerably from other FMD viruses. As already pointed out potent vaccines have been successfully produced only from a few strains of the SAT serotypes. Therefore a reappraisal of conditions necessary for their successful *in vitro* cultivation and for their stabilization during the subsequent stages of vaccine manufacture may be necessary before successful vaccines can be consistently produced against other than exceptional strains of these serotypes.

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