Improved Technique for the Isolation of Temperature-Sensitive Mutants of Foot-and-Mouth Disease Virus

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An improved temperature-shift selection and screening method is described for the isolation of temperature-sensitive mutants of foot and mouth disease virus.

The isolation of temperature-sensitive (*ts*) mutants of foot-and-mouth disease virus (FMDV) for genetical analysis and physiological determination of viral gene products has previously involved screening large numbers of randomly isolated plaques from mutagenized virus stocks (1, 4) and has therefore required large quantities of tissue culture and considerable labor. Selection procedures exploited in other virus systems (3) have increased the recovery frequency of such mutants. This report describes a selection technique by temperature shift which permits the identification of *ts* mutants by plaque morphology, and a simplified screening procedure which permits a large number of isolates to be screened quickly with minimal tissue culture requirements.

The viruses used were FMDV type 0, strains 1 and 1860, and FMDV type SAT 2, strain SA 2/67. BHK-21 cells were used throughout. The selection procedure involved limit-dilution growth of mutagenized virus in agar-cell suspension cultures. The cultures were a modification of those described by Cooper (2) and consisted of a basal layer (5 ml of LYH medium supplemented with 10% bovine serum and 0.6% agarose in 6-cm plastic petri dishes) with a second layer of 1 ml of freshly trypsinized BHK-21 cells (1.2×10^7 cells/ml) in LYH medium, 0.2 ml of the appropriate virus dilution mixed with 1.2 ml of LYH medium supplemented with 10% bovine serum and 1.2% agarose. The infected cultures were incubated at 37 C (permissive temperature) for 24 h in a humidified atmosphere containing 5% CO₂ to initiate plaque formation. A staining overlay containing 2 ml of equal volumes of 0.1% 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) and 1.5% glucose in 0.9% sodium chloride with 0.6% agarose was

added, and the cultures were transferred to 41 C (restrictive temperature) for a further 24 h in sealed plastic boxes in a water bath with accurate temperature control. The plaques were of two types: large, haloed plaques, which constituted 99.9% of the population, and small, clear-lysis plaques. This morphological difference was enhanced by the three-dimensional nature of the assay system. On isolation and retesting, the small, clear-lysis plaques were found to be *ts* mutants. Thus, the *ts* mutants were able to initiate plaque formation at 37 C, but replication was inhibited when transferred to the higher temperature. The wild-type viruses, however, were able to continue replicating at the restrictive temperature with partial lysis at the circumference of the plaques, resulting in the haloed appearance (Fig. 1). These clear-lysis plaques were isolated, cloned by normal procedures, and screened for temperature sensitivity on BHK monolayers grown in Flow microtiter plastic plates (M-29 ART). The monolayers were prepared by adding 0.1 ml of a trypsinized cell suspension into each well (4×10^5 cells/ml in Eagle medium supplemented with 5% bovine serum, 10% tryptose phosphate broth, and antibiotics) and incubated at 37 C for 24 h in a humidified atmosphere containing 5% CO₂. The monolayers were washed with phosphate-buffered saline (0.15 ml/well) and infected with 0.025 ml of appropriate virus dilutions, and the virus was allowed to adsorb for 30 min at 37 C before being overlaid with Eagle medium (0.15 ml/well) and sealed with Celluloid tape. Replica plates were incubated for 24 h at permissive (incubator) and restrictive (water bath) temperatures, and the monolayers were fixed and stained with 10% saturated methylene blue in 0.4% formaldehyde. The titre by cytopathic effect was compared at

FIG. 1. Agar-cell suspension culture infected with a mixture of FMDV type 0, strain 1, wild-type, and *ts* mutant virus. After the temperature selection, the plate was further stained with 0.3% toluidine blue to enhance photographic contrast.

FIG. 2. Replica plates of a titration of 24 clear-lysis plaques at the permissive (37 C) and restrictive (41 C) temperatures. The plates are divided into two, with 4 $\times$ $\log_{10}$ dilutions of the isolates titrated vertically.

FIG. 3. Microtiter plate of a guanidine resistance titration. Duplicate titrations were done, with rows 1, 2, 5, 6, 9, and 10 being overlaid with Eagle medium and rows 3, 4, 7, 8, 11, and 12 overlaid with Eagle medium supplemented with guanidine hydrochloride (400 $\mu\text{g}/\text{ml}$). The normal wild-type virus is titrated in rows 1-4 and two guanidine-resistant mutants are titrated in rows 5-8 and 9-12.

both temperatures and isolates with a 2 $\log_{10}$ difference in titre were kept as *ts* mutants (Fig. 2). Although not fully quantitative, this screening procedure was found to give titres essentially similar to conventional plaque assays and has been extended to screening for other markers such as guanidine hydrochloride resistance (Fig. 3).

The isolation frequency of *ts* mutants by conventional techniques is low (1% or less) and both laborious and costly in terms of tissue culture requirements and reagents. This procedure gave an isolation frequency of 80% (for 38 strain 1 *ts* mutants) with minimal tissue culture requirements. However, whereas conventional techniques involve the screening of randomly isolated plaques grown in the absence of selective pressure (after growth at the permissive

temperature), it is possible that a higher proportion of mutants isolated by this procedure might contain multiple *ts* lesions. This is under investigation at present by means of genetic mapping.

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