# MICROTITER NEUTRALIZATION TEST FOR THE STUDY OF FOOT-AND-MOUTH DISEASE ANTIBODIES

Maria Elma V. Ferreira*

BRIEF REPORT

A microneutralization test (MNT) for foot-and-mouth disease (FMD) virus was described by Wagner and McVicar (4). The authors used PK15, IB-RS-2 and BHK-21 clone 13 cell lines as well as primary cultures of bovine thyroid and kidney cells. In their system, virus-serum mixtures were incubated in tubes, cells were added to these mixtures and the suspension distributed over the wells of disposable plates. The plates were incubated at 37° C in a humidified atmosphere with 5% $CO_2$ and then read microscopically for cytopathic effects after 48 hours of incubation. BHK-21 cells were found to be the most suitable for this system.

In South American FMD laboratories, including the Pan American Foot-and-Mouth Disease Center (PAFMDC), the serum neutralization test in tubes is used routinely. In this paper we propose some changes in the MNT which make it possible for it to replace the tube test and save considerably on materials, time and labor. The MNT also allows for the testing of larger number of sera than has been possible with the tube test. It will have similar advantages over the mouse protection test.

For the MNT, BHK-21 clone 13 (3) or IB-RS-2 (1) cell cultures were dispersed with 1% trypsine (pH 7.7) and suspended in modified Eagle medium (MEM) (3), with 10% bovine serum. Cell suspensions containing 300,000 cell/ml were distributed in 0.1 ml volumes over the wells of disposable microtiter plates**, which were covered with glass plates to allow air circulation. The plates were incubated at 37° C in a $CO_2$ incubator. After 24 h, the monolayer cells were ready for use.

FMD virus strains $O_1$ Campos, $A_{24}$ Cruzeiro and $C_3$ Resende were used in the 4th BHK-21 cell passage. The virus suspensions in MEM had previously been clarified by centrifugation and titrated and stored in 2 ml portions at -70° C. Each portion contained sufficient virus to produce in a one-step dilution the virus suspension needed for a daily test run.

Sera from vaccinated, non-immunized or convalescent cattle were stored at -20° C. They were inactivated at 56° C for 30 min before being tested.

The MNT were made by mixing 0.5 ml of serum dilutions with an equal volume of the virus suspension containing an estimated 1,000 $CCID_{50}$. All dilutions of serum and virus were made in MEM with 250 IU of Penicillin, 25 mg of streptomycin and 25 mg of

** Microtiter Plate FALCON 3040.

* Pan American Foot-and-Mouth Disease Center, Caixa Postal 589-ZC-00, Rio de Janeiro, RJ, Brasil.

Fungizon*. The pH of the medium (2) was adjusted to 7.2 with a solution of 28 mM of HEPES**. The serum-virus mixtures were kept at 37° C for 1 h.

The growth medium was removed from the plates by inverting and vigorously shaking the plate. The surface was dried with commercial paper tissue. The virus-serum mixtures were then distributed in 0.1 ml volumes containing 100 $CCID_{50}$ over the wells of the plate, which were again covered with glass plate and further incubated for 48 h in a $CO_2$ incubator. After that period the cultures were fixed and stained by submersion during 15 min in a solution of 5% formalin with 0.1% crystal violet (Fig. 1).

After rinsing with tap water the plates were dried and ready for macroscopic reading. The dilution which protected 50% of the monolayers was calculated by the method of Spearman-Kärber.

No difference in virus titers was observed between BHK-21 and IB-RS-2 cells, but with IB-RS-2 cells the reading of the positive or negative wells was easier, because of more extensive disruption of the cell layer with virus replication (Fig. 1). For this reason IB-RS-2 cells were preferred for routine tests. Moreover, these cells can be grown at low cost in Earle's medium which is available to laboratories with limited resources.

The use of pre-formed monolayers allowed for a better reproducibility of results than the method described by Wagner and McVicar, probably because the pre-formed monolayers were more uniform and those variables eliminated which occur during the first hours of monolayer formation. The use of HEPES buffer is likely an important factor in the reproducibility of results since it stabilizes the pH during the critical hours of cell attachment and initiation of the monolayers.

Another important factor for obtaining reproducible results is the use of a virus stock stored at -70° C which can be diluted in one-step to prepare the virus suspension needed for each daily test run.

The reproducibility of the MNT was established by a series of tests with 14 cattle sera. Five replicate tests were made from each serum using 6 wells per dilution. Under these conditions a 95% probability exists that the result will vary from the true value by $\pm 0.22$ for viruses $O_1$ and $C_3$, and $\pm 0.35$ for virus $A_{24}$.

No significant differences were observed in serum titers with virus doses varying between 50 and 200 $CCID_{50}$. It was also shown that the mean virus dose during the 6-month test period was $118 \pm 22$, $100 \pm 18$ and $135 \pm 22$ for types O, A and C, respectively, with a 95% confidence limit.

However, virus titers proved to be reduced by at least 50% when cells were used which had recently been grown from stock stored in liquid nitrogen. Therefore such cultures were always given 5-6 passages before use in the MNT and never until normal virus titers were obtained.

No major problems were encountered with the reading of plates when small-plaque-forming viruses were used (Fig. 1 b).

The MNT described in this paper does not require any special equipment except for a $CO_2$ incubator and microtiter plates. Even though the plates are disposable, they can be reused several times. However, they must be cleaned carefully, without scratching the botton of the well.

Further studies are needed to determine the sensitivity and specificity of the MNT in relation to the immune status of cattle. However, the experience to date with the test shows that it is possible to obtain information quickly regarding the value of vaccines used in the field and to carry out serological surveys on large numbers of sera.

* Squibb.

** N-2-Hydroxyethylpiperazine-N'2-Ethanesulfonic Acid.

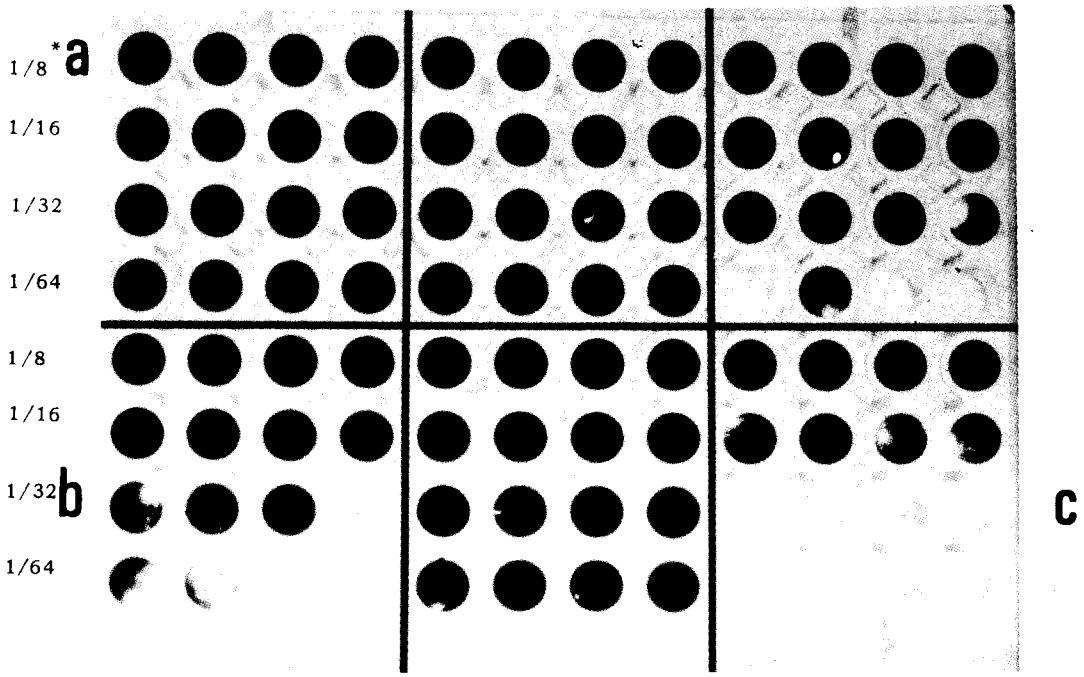


FIGURE 1. Microneutralization test with IB-RS-2 cells. Four dilutions of sera were used divided over 4 wells, permitting the assay of 6 sera per plate. a) The presence of a stained complete cell sheet indicates the existence of sufficient antibodies to neutralize 100 $CCID_{50}$; b) plaque formation, and c) total disruption of the cell sheet indicating the absence of antibodies.
* Serum dilution.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Paul Sutmöller for his stimulation and support and Dr. Vicente Astudillo for the statistical analysis.

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