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

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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% CO₂ 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% trypsin (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 CO₂ incubator. After 24 h, the monolayer cells were ready for use.

FMD virus strains O₁ Campos, A₂₄ Cruzeiro and C₃ 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 CCID₅₀. 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.

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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 CCID₅₀ over the wells of the plate, which were again covered with glass plate and further incubated for 48 h in a CO₂ 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 ±0.22 for viruses O₁ and C₃, and ±0.35 for virus A₂₄.

No significant differences were observed in serum titers with virus doses varying between 50 and 200 CCID₅₀. It was also shown that the mean virus dose during the 6-month test period was 118±22, 100±18 and 135±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 CO₂ 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 bottom 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.
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REFERENCES

1. CASTRO, Maria Pereira de
2. HALLIBURTON, B.S.; BECKER, M.E.

3. MACPHERSON, I.; STOKER, M.

4. WAGNER, G.G.; McVICAR, J.W.