Detection of Foot-and-Mouth Disease Virus Antibodies

I. "Passive" Hemagglutination Test

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A passive hemagglutination test has been developed to detect and measure foot-and-mouth disease virus (FMDV) antibody by using glutaraldehyde as a coupling reagent. An optimal concentration of 10 to 40 μg of virus per ml with 0.25% glutaraldehyde at 25°C for 1 hr was established for the sensitization of sheep erythrocytes. A reaction time of 18 hr at 4°C or 2 hr at 37°C induced good agglutination in the presence of specific antibody. Sensitization was carried out in phosphate buffer, whereas agglutination and preadsorption of nonspecific agglutins from sera were performed in gelatin (0.1%, w/v)-stabilized, phosphate-buffered saline. An optimal pH of 7.2 was also established for all reactions. Antibodies derived from guinea pigs hyperimmunized by infecting with FMDV, types A, O, and C were both virus- and type-specific. Preliminary experiments showed that strain A-119 and strain A-24 Cruzeiro could also be distinguished by hemagglutination. Parallel hemagglutination and complement-fixation tests showed the former to be two to four times more sensitive than the latter.

For rapid diagnosis of disease, it is important to use techniques sufficiently sensitive to detect antigens or antibodies in animal sera. Detection of antibodies in animal sera is often the only way to test for the occurrence of foot-and-mouth disease virus (FMDV) in the field. Although complement-fixation (CF) and neutralization tests are sensitive, they lack simplicity and speed, respectively. It was felt that a hemagglutination test could overcome many of these difficulties. Michelsen and Bachrach (10) and Singh et al. (14) failed to obtain significant hemagglutination-inhibition activity with FMDV free from nonspecific effects. Hence, a passive hemagglutination (HA) method was investigated.

The conjugation of proteins to erythrocytes has proved a valuable tool for identifying traces of antibodies in such fluids. Many hemagglutination techniques involve either direct adsorption of antigens to erythrocytes or their attachment by covalent coupling compounds such as tannic acid. However, these compounds often disrupt the cells before antigens can be attached. Consequently, the use of a stabilizing reagent such as formaldehyde has been necessary before sensitization with a coupling compound could occur (6, 7).

Recently, Avremeas (1), Bing et al. (3), and Onkelinx et al. (11) found glutaraldehyde to be useful in coupling proteins to erythrocytes in the HA test. Moreover, this reagent acts as a stabilizer as was first reported by Ling (8) so that erythrocytes require no pretreatment with formaldehyde.

This report describes an HA test with glutaraldehyde to detect FMDV antibodies in animal sera.

MATERIALS AND METHODS

Glutaraldehyde. Experiments were carried out with stock solution of 25% aqueous glutaraldehyde (Fisher Scientific Co., New York, N.Y.).

Diluents. The sensitization of erythrocytes with antigen was performed in the presence of phosphate buffer (PB; 0.15 M Na2HPO4, 7H2O; 0.15 M KH2PO4 at pH 7.2) by the method of Herbert (6). All other operations were carried out in phosphate-buffered physiological saline containing 0.1% (w/v) gelatin (PBSG, 0.15 M Na2HPO4, 7H2O; 0.15 M KH2PO4, 0.15 M NaCl at pH 7.2).

Antigens. Erythrocytes were sensitized with FMDV (type A, strain 119; type A, strain 24 Cruzeiro; type O, strain Caseros; type C, strain Rezende) which

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were produced by the procedure of Polatnick and Bachrach (13). The virus was purified as previously described (2). Its concentration was measured by spectrophotometry assuming an extinction coefficient of $E_{260\text{nm}}$ = 76. The purified virus was dialyzed against, stored in, and subsequently diluted with 0.2 M NaCl containing 0.05 M sodium phosphate, pH 7.5 (2).

Other tests were performed with infective guinea pig vesicular fluid (GPVF) obtained by infecting the footpads with the above FMDV types and harvesting at maximal lesion activity.

Antisera. Antisera were obtained from hyperimmunized guinea pigs after infecting them with FMDV in the footpads. One to 3 months later, they were inoculated intramuscularly with 1.0 ml of 10% FMDV infective GPVF, and blood samples were obtained 10 days later (4).

Bovine and swine sera immune to FMDV, A-119 were obtained from accumulated supplies but none were hyperimmune.

Virus specificity tests were carried out with sera from (i) swine immunized with vesicular exanthema (type A-48); (ii) cattle immunized with rinderpest (type Kabete O); (iii) goats immunized with African horse sickness (type 9); (iv) guinea pigs immunized with vesicular stomatitis (type Indiana C); and (v) cattle immunized with infectious pustula vulvovaginitis.

All antisera were heated at 56°C for 30 min to inactivate complement before use.

Preparation of antisera. Nonviral specific agglutinins of erythrocytes were preabsorbed from the sera by adding to one volume of serum, one volume of PBSG, and two volumes of a 20% suspension of erythrocytes. The mixture was agitated every 15 min during the 60 min of incubation in the water bath at 37°C. Six volumes of PBSG were added and the cells were pelleted by centrifugation at 510 × g for 10 min in the rotor (model 277) of a centrifuge (International Equipment Co., Boston, Massachusetts). The supernatant fluid was decanted and stored at -20°C.

Source of erythrocytes. Blood was collected from sheep or other animals into an equal volume of Alsever’s solution [dextrose 2.05% (w/v), sodium citrate 0.8% (w/v), sodium chloride 0.42% (w/v), citric acid 0.0055% (w/v), in distilled water] and stored at 4°C. Immediately before use, 10 ml of this suspension was dispensed into a tapered glass tube and mixed with 40 to 50 ml of phosphate-buffered saline (PBS) and centrifuged for 10 min at 510 × g. The supernatant fluid was decanted and the washing procedure repeated two times.

Sensitization of erythrocytes. To two volumes of virus (40 µg/ml) or a twofold dilution of GPVF was added one volume of a 10% sheep erythrocyte suspension in PB (pH 7.2) followed by one volume of 1% glutaraldehyde in PB which was freshly prepared from stock solution in each test. The mixture was agitated every 15 min during incubation for 60 min at room temperature (25°C). The sensitized erythrocytes were then washed twice with PBSG (pH 7.2) and centrifuged to a pellet at 510 × g for 10 min. After removal of supernatant fluid, the erythrocytes were finally resuspended in 20 volumes of PBSG for a 0.5% suspension.

Standard HA test. Serial twofold dilutions of the antisera were prepared in PBSG and 0.3-ml portions were transferred to a series of test tubes (13 by 100 mm). To each tube, 0.3 ml of a suspension of sensitized erythrocytes was added. The mixtures were shaken once and then incubated for 18 hr at 4°C or 2 hr at 37°C. The titer was defined as the reciprocal of the highest antisera dilution still giving a definite agglutination pattern. Tubes were designated + for definite agglutination or — for definitely no agglutination. Marginal tubes were designated by ± (Fig. 1).

Inhibition of HA test. The procedure consisted of mixing 0.15 ml of antigen (160 µg/ml) with 0.15 ml of twofold dilutions of the sera and incubating at 37°C for 30 min. Excess antibody was assayed by adding 0.3 ml of sensitized erythrocytes.

CF test. This test was carried out by the method of Cowan and Trautman (5) by using that dilution of antigen giving maximum fixation of complement.

Agar gel precipitin reactions. The double-diffusion technique used was essentially that described by Ouchterlony (12) but employing 1% (w/v) washed agar in pH 8.6 barbital-acetate buffer.

RESULTS

Development of the HA test. Preliminary experiments with formaldehyde as a stabilizing reagent for bovine or sheep erythrocytes and tannic acid as coupling compound for FMDV, A-119 produced inconsistent results and poor sensitivity. Several positive reactions with glutaraldehyde as coupling reagent, however, justified further investigations into the optimal conditions for a practical test.

For coupling virus to sheep erythrocytes in the presence of 0.25% glutaraldehyde, the pH range was varied between 6.0 and 8.0. An optimal pH level of 7.2 was found suitable and the reaction was complete in 60 to 180 min at 25°C. Under these conditions, optimal agglutination of sheep erythrocytes was independent of pH in the range tested (6.4 to 8.0). The ability of sensitized erythrocytes to agglutinate in the presence of specific antibody was determined at 4°C (18 hr), 25°C (3 hr), and 37°C (2 hr). Best agglutination patterns were obtained at 4°C although comparable results were obtained at 37°C. This finding may be important for rapid diagnostic procedures.

Several different soluble salts were independently tested for their ability to enhance the agglutination reaction, by including them in PB. The presence of 0.9% (w/v) NaCl gave the best results and was subsequently used in all tests reported here. The inclusion of 0.1% gelatin in PB stabilized hemagglutination and produced more consistent results. Samples of FMDV, A-119 were treated with 0.06 to 0.25% glutaraldehyde and were tested for their ability to precipi-
tate specific antibody after immunodiffusion in gels. No precipitin lines were visible with any sample. Thus, it was concluded that the virus had been denatured. Nevertheless, since 0.25% glutaraldehyde was the minimum concentration found to preserve reaction stability and maximum titer, this concentration was employed at all times.

Next, erythrocytes from a number of hosts were tested for their ability to agglutinate with the same serum and virus. Sheep erythrocytes gave the best results of those tested, followed closely by those from swine. Erythrocytes from chickens, goats, cattle, and horses produced very poor reactions.

All preliminary tests were made with purified A-119 (140S) virus so that optimal concentrations for maximal agglutination could be accurately measured (2). Table 1 shows that virus concentrations in the range 10 to 40 µg/ml produced good agglutination free from unacceptable non-virus-specific agglutination. Nevertheless, higher virus concentrations could prove useful for detecting trace amounts of specific antibody in animal sera.

Inactivated virus, unconcentrated infective tissue culture fluid, and GPVF were also tested. Formaldehyde (0.1%, v/v)-inactivated virus induced slightly less, and N-acetylatedenimine [1-acetilaziridine (0.05%, v/v) donated by Dow Chemical Co., Freeport, Tex.] inactivated virus induced greater agglutination than control non-inactivated virus. Whereas infective GPVF induced variable agglutination reactions, infective (A-119) baby hamster kidney tissue culture fluid produced negative results. This fact was perhaps due to the low antigenic content of the fluid.

Figure 1 shows an example of an experiment in which bovine antiserum to FMDV, A-119 was used. This particular experiment was carried out by using a white ceramic tile to aid photography, containing shallow circular depressions (2.2 mm in diameter, 0.8 mm deep).

Table 2 shows the data obtained from an HA test in which infective GPVF was used as a source of antigen. In this case, homologous titers were high compared to heterologous and non-specific titers. The use of infective GPVF would be advantageous for diagnostic purposes in which many antigens of high concentrations are required without the inconvenience of preconcentrating them.

The conditions established for an HA test were considered sufficiently optimal at this stage to warrant further evaluation of disease and type-specific reactions.

**Virus specificity.** The HA tests, designed to detect the presence of antibody in antisera from various hosts infected with vesicular exanthema,

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**TABLE 1. Effect of sensitizing sheep erythrocytes with different concentrations of purified foot-and-mouth disease virus (FMDV), type A-119**

| Pure FMDV A-119 | Bovine antiserum to FMDV, A-1, at dilutions of | Normal bovine serum at dilutions of | Buffer replacing serum |
|-----------------|-----------------------------------------------|-----------------------------------|------------------------|
| µg/ml           | 1/20 1/40 1/80 1/160 1/320 1/640 1/1280       | 1/20 1/40                        |                        |
| 80              | +     +     +     +     +     +     +     | +     +                         | +                      |
| 40              | +     +     +     +     +     +     +     | +     +                         | +                      |
| 20              | +     +     +     +     +     +     +     | +     +                         | +                      |
| 10              | +     +     +     +     +     +     +     | +     +                         | +                      |
| 5               | +     +     +     +     +     +     +     | +     +                         | +                      |
| 0               | +     +     +     +     +     +     +     | +     +                         | +                      |

*a* +, Designates complete agglutination.

b –, Designates no agglutination.

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**FIG. 1. HA test between A-119 virus and bovine antiserum (28 days postinoculation). The reciprocal of the serum dilutions are shown above each well and the titers are shown below the patterns according to +, ±, − designation. Symbols: +, complete agglutination; −, no agglutination; ±, intermediate reactions; ns, normal bovine serum; n, buffer replaces normal bovine serum; V-GSC, virus-glutaraldehyde-sensitized erythrocytes; N-GSC, no virus-glutaraldehyde-sensitized erythrocytes.
rinderpest, African horse sickness, vesicular stomatitis, and infectious pustular vulvovaginitis were carried out using FMDV, types A, O, and C as antigens. All HA titers were <10 except in the presence of homologous antisera. The HA test is, therefore, capable of distinguishing antibody specific to the virus causing the particular disease state.

Type specificity. In Table 3 are compared the HA and CF titers of three types of FMDV in the presence of homologous and heterologous antisera. It is evident that homologous antisera demonstrate strong type-specificity in both tests but the HA test is two to four times more sensitive for detecting antibody than the CF test, although 10 to 100 times more virus was used in the HA test. It was difficult to compare heterologous reactions because of low titers.

In Table 4 the titers obtained by the inhibition of HA test are compared. The type specificity shown by this test was comparable with that of the HA test (Table 3).

Strain specificity. In Table 5 are compared the HA titers of different virus strains in the presence of homologous and heterologous antisera. All antigens in this test were derived from GPV. A high level of specificity with little cross-reaction was apparent.

### DISCUSSION

Of the various factors that play a role in the coupling reaction with glutaraldehyde, the most important are glutaraldehyde concentration, pH level, and antigen concentration. The reaction time and temperature have little effect on the activity. This finding varies from the results of Onkelinx et al. (11), who found that the antigen concentration had little effect, although this result may have been because of differences in

#### TABLE 2. Passive hemagglutination reactions between foot-and-mouth disease virus (FMDV) types in guinea pig (GP) vesicular fluid

| GP antiserum | FMDV type | A | O | C | No virus |
|--------------|-----------|---|---|---|---------|
| Anti-A       | <20       | 80 | <20 | 20 | <20     |
| Anti-O       | <20       | 20 | 80 | 20±| <20     |
| Anti-C       | <20       | 160| <20| 80 | <20     |

* Expressed as the reciprocal of the highest antiserum dilution still giving a definite agglutination pattern minus the titer obtained with normal GP serum.

#### TABLE 3. Comparison of the sensitivities of the passive hemagglutination (HA) and complement-fixation (CF) tests by using guinea pig (GP) antisera to foot-and-mouth disease virus (FMDV), types A, O, and C

| GP antiserum | FMDV type | HA | CF | HA | CF |
|--------------|-----------|----|----|----|----|
| Anti-A       | 320       | 111| 20 | 10 | 20 |
| Anti-O       | 10        | 640| 285| 10 | 20 |
| Anti-C       | 10        | 20 | ±  | 640| 167|

* Expressed as the reciprocal of the highest antiserum dilution still giving a definite agglutination pattern minus the titer obtained with normal GP serum.

** Fifty per cent CF end point (C'H_{50}).

#### TABLE 4. Inhibition of passive hemagglutination test by preadsorption of foot-and-mouth disease virus (FMDV) specific antibody in guinea pig (GP) antiserum with FMDV, types A, O, and C

| GP antiserum | Erthrocytes sensitized with FMDV type | FMDV type used for inhibiting antibody | No virus | A | O | C |
|--------------|--------------------------------------|---------------------------------------|---------|---|---|---|
| Anti-A       | A                                    | A                                     | 160     | <20| 160| 80|
| Anti-O       | O                                    | O                                     | 320     | 320| 40 | 320|
| Anti-C       | C                                    | C                                     | 640     | 640| 640| 20|

* Expressed as the reciprocal of the highest antiserum dilution still giving a definite agglutination pattern minus the titer obtained with normal GP serum.

#### TABLE 5. Passive hemagglutination reactions between foot-and-mouth disease virus (FMDV), strains A-119 and A-24 Cruzeiro in guinea pig (GP) vesicular fluid, and their GP antisera

| GP antiserum | FMDV antigen | Serum titer |
|--------------|--------------|-------------|
| Anti-A-119   | A-119        | Expt 1      |
| Anti-A-24    | A-24         | <20         | Expt 2      |
| Anti-Cruzeiro| A-119        | 300         |
|              | A-24         | 900         |

* Expressed as the reciprocal of the highest antiserum dilution still giving a definite agglutination pattern minus the titer obtained with normal GP serum.
antigen (bovine serum albumin) and the concentrations used.

The HA test could be used to detect and measure FMDV antibodies in guinea pig, bovine, and swine sera. Good homologous reactions have been obtained only with guinea pig sera. Although some preliminary results showed that this was possible with antibody of bovine or swine origin, positive HA reactions with heterologous sera were more frequent. It is important to consider, however, that all bovine and swine sera tested were either from vaccinated animals or from animals infected once only, whereas all guinea pig sera were hyperimmune. The matter is being investigated further.

Parallel use of the HA test with its high sensitivity and the CF test with its high specificity could be important as a diagnostic procedure for detecting FMDV antibodies. As yet, the relative sensitivities of the neutralization and HA tests have not been directly compared in the present system, but results obtained with poliovirus (9) using formaldehyde and tannic acid show the neutralization test to be a little more sensitive.

The relatively high sensitivity, simplicity, and short duration of the HA test compared to the neutralization and CF tests suggest its use as a diagnostic tool. In practice, a pool of several virus types could be used to detect a range of specific antibodies in animal sera. However, as the results show, it may be necessary to include several antigenic strains in the mixture for more precise antibody identification.

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