Complement-Fixation Analysis of Four Subtypes of Foot-and-Mouth Disease Virus Type A

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Complement-fixation patterns were established for four subtypes of foot-and-mouth disease virus by block assays against homologous and heterologous antiserum. Inhibition of fixation by excess antigen was observed in most homologous systems but rarely in the heterologous systems. The heterologous antibody titers were, in all instances, considerably lower than those for the homologous systems. Although relatively high dilutions of antiserum may be desirable for subtyping, higher concentrations of antibody should be used for determining serological types.

The complement-fixation (CF) technique provides a useful tool for differentiating the seven recognized serological types of foot-and-mouth disease virus (FMDV), as well as approximately 62 subtypes within these serotypes. The recognition of different FMDV subtypes is of vital concern for vaccine preparation and evaluation of efficacy under field conditions.

The CF test was first used by Ciucu (4) for typing guinea pig-adapted FMDV of bovine origin. Traub and Möhlmann (14) used this test to demonstrate antigenic differences between three FMDV strains of guinea pig-adapted virus, and drew attention to the possible significance of these observations to selection of vaccine strains. As a result of comparisons between three strains of virus type A, strain 119 (A-12 Pirbright) and strains MP and MI from the Mexican outbreak of 1947 to 1949, Galloway et al. (7) found that vaccinated cattle withstood challenge better with the same subtype of virus present in the vaccine than against heterologous subtypes. Brooksby (3) confirmed the findings of Traub and Möhlmann (14) and introduced his adaptation of the method of Wadsworth and Maltaner (15) to the study of FMDV using the amount of complement fixed as a measure of differences in titer of immune sera reacting with homologous and heterologous antigens.

Improvements of techniques were introduced by Bradish et al. (2), Bradish and Brooksby (1), Graves (8), and Davie (6), and the procedure was adopted for studying new FMDV isolates at the World Reference Laboratory, Pirbright, England.

A somewhat different CF procedure was used by Marucci (11) for FMDV studies and was based on that of Osler et al. (12), where the titer of a reagent is represented as the reciprocal of the dilution fixing four of five 50% hemolytic units of complement (CH₅₀). This procedure was used by Cowan and Trautman (5) and Roumiantzeff and Fayet (13) to study isolated antigenic components of FMDV. The procedure is readily amenable to block, or checkerboard, assays where varying dilutions of both antigen and antibody are examined. Block assays provide antibody as well as antigen titers, and permit overall evaluation of antigen-antibody interactions.

This report (taken, in part, from a thesis presented by C. A. Lobo as a partial fulfillment of the requirements for the M. S. degree in Veterinary Science at the University of Wisconsin, Madison, 1973) describes the comparison of four different subtypes of type A FMDV by CF block assays where homologous and heterologous reactions were examined to evaluate conditions for typing and subtyping these viruses.

MATERIALS AND METHODS

Viruses. Four different subtypes of FMDV type A were studied. A-31 (A-Colombia 69) was the cause of an epizootic in the central part of Colombia during 1969 [LOBO (10)]. A-32 (A-Venezuela 70) was isolated in 1970 in the state of Bolivar, Venezuela. A-24, strain Cruzeiro, originated in Brazil. A-12 originated from England.

Antigens. Infectious bovine tongue epithelium was inoculated intradermally (i.d.) into the hind footpads.
of 400- to 500-g guinea pigs. The fluids aspirated from vesicles occurring on the paws 20 to 24 h later were pooled and clarified by centrifugation at 980 × g for 15 min. Several passages were made in guinea pig footpads until each virus was adapted (when secondary lesions developed on the forefeet of 80 to 90% of the inoculated guinea pigs within 72 h [8]). Vesicular fluids were stored at −80°C. An estimate of the mass concentration of each virus could be obtained by multiplying the reciprocal of the antigen dilution titer by 0.231, the value obtained by Cowan and Trautman for the A-12 virus (5). This is based on the assumption that the final titer is obtained with the particles associated with infectivity (140S) and that A-24, A-31, and A-32 viruses will have the same mass of virus fixing four of five CH₄⁺. However, a reliable estimate necessitates the exact determination for each separate virus. In light of the above considerations, estimates of the concentrations would be as follows: A-12, 74 μg/ml; A-24, 37 μg/ml; A-31, 74 μg/ml; and A-32, 28 μg/ml.

**Hyperimmune sera.** Guinea pigs were infected by i.d. inoculation of plantar pads with guinea pig vesicular fluid (GPVF) containing A-12, A-24, A-31, or A-32 virus, followed in 4 weeks by a subcutaneous inoculation of a 10% suspension of homologous infectious GPVF. Guinea pigs were exsanguinated 10 days after the second injection, and blood was collected in 50-ml conical tubes. Serum separated from the clot was pooled, heated at 56°C for 30 min, and stored at −20°C after appropriate serological evaluation. Each pool consisted of approximately 100 individual guinea pig sera.

**CF procedure.** The CF method was the one used by Cowan and Trautman (5) in the study of FMDV systems. Two-dimensional block titrations were performed as follows. A 0.4-ml amount of each twofold dilution of antiserum was pipetted in a series of tubes (13 by 100 mm) to which was added 0.5 ml of guinea pig serum containing 5 CH₄⁺. Twofold antigen dilutions were added so each dilution of antigen was tested against all dilutions of the antiserum, and each dilution of antiserum was tested against all antigen dilutions. Suitable controls of anti- and pro-complementary activity were set up by testing each antigen and antibody dilution against 5 and 2 CH₄⁺. A complement titration was run with each test. After incubation at 37°C for 1.5 h, 0.2 ml of sensitized sheep red blood cells containing 10⁴ cells per ml was added to each tube, and the mixtures were incubated for 1 h at 37°C. The tests were kept at 4°C overnight to permit unlysed cells to sediment. Degrees of hemolysis were determined by visual reading, and results were recorded as 0, 1, 2, 3, or 4, corresponding to 0, 25, 50, 75, and 100% lysis, respectively. Results of such block titrations are graphically presented in Fig. 1 for the subtypes A-12 and A-24. The points represent reaction mixtures giving an estimated 50% lysis. Reactions occurring within the line joining the points showed no hemolysis or less than 50%, whereas reactions outside the line gave complete or greater than 50% hemolysis.

From block titrations, the titer of both antigen and antibody can be calculated. The antiserum titer is

*Fig. 1. Complement-fixation patterns obtained with subtypes A-12 and A-24 antisera tested against type A FMDV subtypes A-12, A-24, A-31, and A-32.*

**Table 1. Complement-fixation results obtained with subtypes A-12, A-24, A-31, and A-32 antiseras tested against homologous and heterologous type A FMDV subtypes**

| Antigen dilutions | Antiserum titer |
|-------------------|-----------------|
|                   | A-12 | A-24 | A-31 | A-32 |
| A-12              |      |      |      |      |
| 1:10              | 120  | 40   | 20   | 20   |
| 1:20              | 320  | 40   | 20   | 20   |
| 1:40              | 320  | 40   | <20  | <20  |
| 1:80              | 160  | 30   | <20  | <20  |
| 1:160             | <20  | <20  | <20  | <20  |
| 1:320             | <20  | <20  | <20  | <20  |
| A-24              |      |      |      |      |
| 1:10              | 40   | 120  | 40   | 20   |
| 1:20              | 40   | 120  | 40   | 20   |
| 1:40              | 30   | 160  | 40   | <20  |
| 1:80              | <20  | 160  | 30   | <20  |
| 1:160             | <20  | 120  | <20  | <20  |
| 1:320             | <20  | <20  | <20  | <20  |
| A-31              |      |      |      |      |
| 1:10              | 30   | 30   | 120  | 20   |
| 1:20              | 40   | 40   | 240  | 20   |
| 1:40              | 40   | 60   | 240  | 20   |
| 1:80              | 20   | 40   | 320  | <20  |
| 1:160             | <20  | 30   | 80   | <20  |
| 1:320             | <20  | <20  | <20  | <20  |
| A-32              |      |      |      |      |
| 1:10              | 30   | 30   | <20  | 60   |
| 1:20              | 30   | 40   | 20   | 80   |
| 1:40              | 40   | 40   | <20  | 120  |
| 1:80              | 40   | 40   | <20  | 120  |
| 1:160             | 20   | 20   | <20  | 40   |
| 1:320             | <20  | <20  | <20  | <20  |

* Fixation time of 1.5 h at 37°C.
* Reciprocal of antiserum dilution giving 50% lysis.
* Less than dilution 1:20.
serum titer (1:320) was obtained with antigen diluted 1:80. Antigen titer may be rather arbitrary and dependent upon the antiseraum dilution used; however, in an ideal system, the antigen titer obtained may, within limits, be independent of serum dilutions utilized. In the example cited above, an antigen titer of 1:160 was obtained with serum diluted 1:20, 1:40, or 1:80. The block assay provides an over-all examination of the system and establishes the optimal concentrations of reagents for subsequent assays.

RESULTS

Four virus subtypes of FMDV were examined by CF block assays with homologous and heterologous antiserum to establish general patterns of reactivity. The results obtained with all homologous and heterologous systems are summarized in Table 1. Reactions obtained with all homologous systems were generally typical in that inhibition of fixation occurred with high concentrations of antigen and, except for the A-32 system, antigen end points tended to cut off sharply. An optimal antigen dilution that gave the maximal serum titer was evident for each homologous system. The maximal homologous serum titer was obtained for A-12 when the antigen was diluted 1:20 or 1:40, with A-32 at a 1:40 or 1:80 dilution of antigen, and with A-24 at a 1:80 dilution of antigen.

Antiserum to A-32 was relatively weak, giving a homologous titer of 1:120. With A-24 serum the titer was 1:160, whereas antisera to A-12 and A-31 titered 1:240.

Heterologous viral systems frequently failed to demonstrate inhibition of fixation with an antigen excess. Antiserum titers against heterologous antigens were considerably lower than those for the homologous systems (Table 1). Although not included in the table, the different antiserum prepared against the various subtypes of virus type A did not show any cross-reaction against other serotypes of FMDV included as controls.

DISCUSSION

There are many procedures for performing CF tests, and selection of conditions used is generally dependent on the type of information sought.

One of the tests most frequently used is that described by Brooksby (3), in which a constant serum dilution, a constant antigen dilution, and a series of several doses of complement are employed. The degree of hemolysis is determined, and the amount of complement fixed is calculated. Differences among types and strains within types are determined according to the amount of complement fixed in each system.

However, the use of fixed amounts of serum and antigen presents certain problems in typing and subtyping the field strains.

Block assays performed in this study evaluated test conditions that would readily differentiate the subtypes studied. It is evident from results presented in Table 1 Fig. 1 that, if a single dilution of antiserum was used for subtyping purposes, a dilution could be selected which would essentially eliminate any cross reactions. For example, A-12 antiserum (Table 1) at a dilution of 1:80 would give good fixation with homologous virus, but none with heterologous subtypes. However, if antiserum was used at a dilution of 1:20, considerable difficulty could be encountered in differentiating the viruses because of the cross-reactions obtained.

Use of relatively high serum dilutions generally does not reduce sensitivity of the CF test for detecting viral antigen. This is illustrated with antiserum to A-12 virus (Fig. 1), reacting with homologous virus where essentially the same antigen titers were obtained with serum diluted 1:20, 1:40, or 1:80.

Although relatively high dilutions of antisera are desirable for subtyping purposes, higher concentrations of antibody should be used for determining virus types, i.e., type A, O, C, etc., classification. For example, antiserum to A-24 virus (Table 1 and Fig. 1) at a 1:20 dilution reacted satisfactorily with all four virus subtypes and could be used to determine the presence of any one of these viruses in a diagnostic sample. This higher serum concentration broadened the spectrum of reactivity with these virus subtypes tested. However, it may be seen (Table 1) with antiserum to A-31 virus that even at a dilution of 1:20 relatively poor reactions were obtained with viruses A-12 and A-32. Consequently, this serum could possibly miss these subtypes if used for typing purposes.

Although use of more dilute serum improved specificity and provided some economy of the antiserum reagents, a theoretical problem exists that should be considered. If a low antibody concentration is used to test a single antigen dilution, inhibition of fixation could occur because of antigen excess. However, in actual practice it is unlikely that samples containing such high levels of antigen would be encountered, and the problem is readily circumvented by using varying dilutions of antigen.

Another problem, pointed out by Kabat and Mayers (9) is that, in titrations of an antiserum with related antigens, heterologous antigen may actually give a higher serum titer than the
homologous antigen if relatively high concentrations of the antigens are used. This supports the view that in comparative studies it is desirable to use several different concentrations of antigens to avoid such errors.

Under the conditions employed in the present study, the CF reactions were probably due to particles associated with infectivity having a sedimentation coefficient of 140S (5). As was shown by Cowan and Trautman, (5), short incubation periods tend to minimize the reactivity of the other viral antigens such as the noninfectious protein subunits of the virus which have a sedimentation coefficient of 12S and an infection-associated antigen or enzymatically inactive viral ribonucleic acid dependent-ribonucleic acid polymerase. It is assumed that the cross-reactions are due to the 140S particles and to a lesser extent to the 12S particles. The latter give poor CF in the short, warm-temperature test, but good fixation in the long, low-temperature test.

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