Detection and Quantification of Foot and Mouth Disease Virus by Enzyme Labelled Immunosorbent Assay Techniques

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SUMMARY

Enzyme labelled immunosorbent assays (ELISA) have been developed to detect and quantify foot and mouth disease (FMD) virus using flexible plastic microtitre plates. The methods were successful for the specific detection of FMD virus and were 50 to 100 times more sensitive than the complement fixation test. The application of the ELISA techniques to FMD virus typing and subtyping, and to the assay of antigen concentration during manufacture of vaccines is discussed.

INTRODUCTION

The micro ELISA test is frequently used for the detection and quantification of viruses; (Voller et al. 1976; Wolters et al. 1976, 1977; Halbert & Anken, 1977; Miranda et al. 1977; Yolkens et al. 1977). The technique had been found to be rapid and can be as sensitive as radioimmunoassay (RIA), and makes it possible to detect small amounts of material without the problems associated with the use of radioactive compounds.

The measurement of bovine antibodies to FMD virus was shown to be possible using the micro ELISA test (Abu-El Zein & Crowther, 1978). In this paper we describe methods used to detect and quantify FMD virus and indicate the applications of the test to problems associated with FMD virus.

METHODS

Viruses. FMD viruses type O/UK 1860/1967 and type A/Allier/1960, were grown in monolayers of BHK 21 cells and purified by sucrose density gradient centrifugation as described by Brown & Cartwright (1963) using 1% SDS instead of deoxycholate. Purified viruses were stored at -70 °C in siliconized glass vials. Swine vesicular disease (SVD) virus UK 27/72 was grown in IBRS<sub>2</sub> cells and purified using the same method as for FMD virus.

Antisera. Antiserum against purified (140S) O/UK/1860/1967 FMD virus was prepared in guinea pigs. Purified virus was inactivated using acetyleneimine (AEI), at a final concentration 0.05%, by incubation at 26 °C for 30 h. One vol. of inactivated virus was emulsified with one vol. of Freund's complete adjuvant (Difco Labs., Detroit, Michigan, U.S.A.) and five guinea pigs each received 50 µg of virus, intramuscularly at two sites. Animals were exsanguinated after 28 days and the sera pooled.

Goat anti guinea antiserum was obtained commercially (Miles Labs. Ltd, U.K.).

The IgG fractions of the antisera were prepared as described previously (Abu-El-Zein &
Crowther, 1978). The IgG concentration was calculated from the absorbance at 278 nm ($A_{278} = 1.4$). Samples were stored at $-20\,^\circ C$ in siliconized glass vials.

Conjugation of enzyme to IgG. Alkaline phosphatase (EC.3.1.3.1., Type VII, Sigma, England) was conjugated to the IgG fraction of the antisera according to the method of Avrameas (1969). The working dilution of each conjugate was determined as described by Voller et al. (1976). Stock conjugates were stored at $4\,^\circ C$ after the addition of 5% ovalbumin (final concentration) and sodium azide (final concentration 0.02%).

Virus detection experiments. Tests were performed using purified virus of known concentration, determined after spectrophotometric analysis at 259 nm of sucrose density gradient material, as described by Bachrach et al. (1964). The approximate particle concentration of purified SVD virus UK 27/72 was determined using the same formula, where this virus was used as a control. The methods described state conditions found to be optimal for the tests based on preliminary studies to determine parameters such as incubation times and temperatures number of washes after each step, addition of different concentrations of Tween 20 and/or ovalbumin. Several important points concerning conditions are raised in the results.

Direct test. Viruses were attached to the solid phase (Flexible polyvinyl microplates, U-bottomed wells, Dynatech, England, catalogue no. 1-220-24), by incubating the plates with varying dilution of purified virus diluted in sodium carbonate/bicarbonate buffer, 0.05 M, pH 9.6, for 3 h at $37\,^\circ C$ (200 µl virus dilutions per well). Unattached virus was then removed by flooding and emptying the wells three times with a solution of saline (0.15 M-NaCl) containing a final concentration of 0.1% (w/v) Tween 20. Each well then received 200 µl of a dilution (pre-titrated) of homologous anti-FMD virus IgG conjugate diluted in PBS with 0.05% (w/v) Tween 20 (PBST), containing 3% final concentration of ovalbumin (Miles Laboratories, Slough, England). Plates were incubated for 1 h at $37\,^\circ C$ while being shaken on a rotary shaking machine (R100 Rotatest, Luckham Ltd, West Sussex, England) at 60 rev/min. Plates were washed three times and 200 µl of substrate was added to each well ($p$-nitrophenyl phosphate, Sigma, England, at 1 mg/ml in 10%, w/v, diethanolamine buffer, pH 9.8). Plates were left for 30 min at room temperature, then the colour reaction stopped by the addition of 50 µl of 3 M-NaOH. Colour readings were made by measuring the absorbance at 405 nm using a Uvichem Spectrophotometer (Hilger and Watts).

Indirect test. Dilution series of purified virus were attached to wells as described above. Homologous guinea pig anti-FMD virus (140S) IgG was then added to a concentration of 2.5 µg/ml in PBST containing 1% ovalbumin (200 µl/well) and the plates were incubated at $37\,^\circ C$ for 1 h with shaking. After washing three times, each well received 200 µl of enzyme conjugated goat anti-guinea pig IgG diluted in PBST containing 3% ovalbumin. Plates were incubated at $37\,^\circ C$ for 1 h with shaking. Substrate additions and incubation procedures were then made as described in the direct test.

Sandwich test. The procedure described by Voller et al. (1976) was followed. Guinea pig anti-FMD virus (140S) IgG was added to wells after dilution in carbonate buffer to 10 µg/ml (200 µl/well). After incubation for 2 h at $37\,^\circ C$ the wells were washed three times and 200 µl of dilutions of purified homologous virus in PBST containing 1% ovalbumin was added. The plates were incubated for 1 h at $37\,^\circ C$ with shaking. After washing three times, 200 µl of enzyme-conjugated anti-FMD virus (140S) guinea pig IgG were added to each well and the plates were incubated at $37\,^\circ C$ for 1 h with shaking. Plates were read after addition of substrate as already described.

For all the methods described, triplicate samples of each virus dilutions were examined. Controls were included to examine the specificity of the tests and are indicated in the results.
Identification of FMDV by ELISA

Results
Attachment of purified FMD virus O1/UK/1860/1967 or antibody to wells was successfully achieved at 37 °C for 3 h although incubation at room temperature overnight gave similar results. Successful assays were also made after drying virus on to wells by leaving plates overnight without lids, or drying in a stream of air. Virus antigens contained in different volumes of diluent (50 μl to 200 μl) were examined, and all gave successful assays; thus 50 μl dilution loops could be used to dilute virus samples in the direct and indirect tests. The use of the rotary shaker increased the reaction of antigen and antibody at the reported stages, presumably by increasing the probability of contact between the reactants; equivalent results were obtained after 3 h at 37 °C on stationary plates. The inclusion of both Tween 20 and ovalbumin where stated was essential to minimize non-specific effects.

Results for the titration of purified type O1/UK/1860/1967 FMD virus, using the three ELISA techniques outlined, are shown graphically in Fig. 1. SVD virus UK 27/72, was used as an antigen control as it had been found to attach to wells in a similar way to FMD viruses and can be titrated using ELISA techniques with antiserum to SVD. The limits for the detection of O1/UK/1860/1967 FMD viruses were similar using all three methods. These were determined as the last well showing a colour reaction reading above the background control readings with suitable confidence limits. The standard deviation from the mean of each assay point was always very low and the end-points were read as illustrated on the specific titration curves on Fig. 1. All three methods were specific in measuring only type O1/UK/1860/1967 virus using the described antisera; neither SVD virus nor type A FMD virus gave colour development under the same test conditions. Fig. 2 and Table 1 show the results of titrating a narrow range of virus concentrations using the sandwich-ELISA
Fig. 2. Quantification of purified type O1/UK/1860/1967 FMD virus by sandwich ELISA. ●—●, Titration of O1/UK/1860/1967. Dashed lines indicate maximum control absorbance levels for: 1 – FMD virus type A/Allier/1960; 2 – SVD virus UK 27/72; 3 – blank wells.

Table 1. Titration of FMD virus type O1/UK/1860/1976 by sandwich ELISA technique*

<table>
<thead>
<tr>
<th>Virus concentration (ng/ml)</th>
<th>1</th>
<th>3</th>
<th>3</th>
<th>Mean</th>
<th>s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>1.402</td>
<td>1.397</td>
<td>1.410</td>
<td>1.403</td>
<td>0.007</td>
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<tr>
<td>175</td>
<td>1.202</td>
<td>1.260</td>
<td>1.302</td>
<td>1.255</td>
<td>0.050</td>
</tr>
<tr>
<td>150</td>
<td>1.102</td>
<td>1.191</td>
<td>1.165</td>
<td>1.153</td>
<td>0.046</td>
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<tr>
<td>125</td>
<td>1.116</td>
<td>1.015</td>
<td>1.003</td>
<td>1.045</td>
<td>0.062</td>
</tr>
<tr>
<td>100</td>
<td>0.950</td>
<td>1.008</td>
<td>0.907</td>
<td>0.955</td>
<td>0.051</td>
</tr>
<tr>
<td>75</td>
<td>0.892</td>
<td>0.889</td>
<td>0.729</td>
<td>0.854</td>
<td>0.063</td>
</tr>
<tr>
<td>50</td>
<td>0.710</td>
<td>0.794</td>
<td>0.710</td>
<td>0.738</td>
<td>0.048</td>
</tr>
<tr>
<td>25</td>
<td>0.614</td>
<td>0.599</td>
<td>0.630</td>
<td>0.614</td>
<td>0.016</td>
</tr>
<tr>
<td>SVD (200≤ ng/ml)</td>
<td>0.335</td>
<td>0.301</td>
<td>0.321</td>
<td>0.319</td>
<td>0.017</td>
</tr>
<tr>
<td>FMD type A (200 ng/ml)</td>
<td>0.485</td>
<td>0.480</td>
<td>0.450</td>
<td>0.473</td>
<td>0.016</td>
</tr>
<tr>
<td>Blank</td>
<td>0.306</td>
<td>0.341</td>
<td>0.312</td>
<td>0.320</td>
<td>0.018</td>
</tr>
</tbody>
</table>

* Regression coefficient of O1/UK/1860/1967 titration line = 0.9815.

technique, in order to assess the relationship of virus dilution to colour development. A linear relationship was obtained over the range of 25 to 200 ng examined. The small standard deviation for each assay point is illustrated in Table 1. The line in Fig. 2 was fitted using linear regression statistics; the regression coefficient was 0.9815. Similar plots were obtained for the direct and indirect techniques using equivalent ranges of virus concentrations.

Similar assays have been performed to titrate FMD virus types A, C, and SAT 1, and all
Confirmation of FMDV by ELISA

Identification of FMDV by ELISA

confirm the test as being type specific under the conditions described, using purified viruses.

Titration curves were reproducible when the same antiviral antibodies and conjugated antisera are used. Tests performed three months apart produced identical titration curves where the reactants had been stored as described.

DISCUSSION

The ELISA technique had been successfully applied to the quantification of purified FMD viruses. The three methods described showed similar limits for the detection of virus. Cumulative data indicate a range of 5 to 30 ng/ml FMD virus may be measured distinct from background controls. This level can be compared to the complement fixation (CF) test which had been reported to detect approx. 1 μg/ml of purified FMD virus (Garland et al. 1977). The methods were specific for type O FMD in this study; SVD and type A FMD virus did not interfere.

The specificity and reproducibility of the assays indicate that standard curves relating known virus concentrations to colour development, using defined reagents, can be constructed in order to assay unknown virus concentrations.

The three methods utilizing the ELISA technique are being examined for different applications to FMD virus research. The sandwich technique is now being used successfully in this laboratory to assess the mass of 140S virus in the presence of 12S and tissue culture material, using antisera prepared against inactivated purified FMD viruses. This is useful in the examination of the quantity of antigenic material to be incorporated in vaccines and eventually it is hoped to produce an automatic system for the quantification of 140S virus throughout the preparative procedures in vaccine manufacture.

The indirect technique, where only a single anti-species conjugate is needed, is being used to detect and type FMD viruses form epithelial samples sent to the World Reference Laboratory, Pirbright. Results are being compared to the conventionally used CF test. Preliminary studies indicate that the increased sensitivity of the ELISA technique over the CF test (50 to 100 times), the lack of anti-complementary factors and the high specificity of the test will make it a useful method for the routine diagnosis of FMD virus.

The direct test is being used to examine subtype differences between FMD virus strains using competition assays.

Studies on the application of ELISA tests to FMD virus research will probably modify the techniques further to deal with specific problems. The versatility of the ELISA when applied to other animal viruses is indicated by the fact that the direct and indirect tests have recently been used to develop assays to quantify viruses as different as African swine fever and SVD in this laboratory.

REFERENCES


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