Establishment of a typing enzyme-linked immunosorbent assay for foot and mouth disease antigen, using reagents against viruses endemic in Thailand


Summary: Antisera were produced at a central laboratory in Thailand against the endemic serotypes (O, A and Asia 1) of foot and mouth disease (FMD) virus. At a regional veterinary laboratory, these antisera were used in an indirect sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and serotyping of FMD virus (FMDV) antigen. ELISA readings of <0.10 optical density (OD) units were considered negative. This was verified using fifty tissue samples which were known to be negative for FMDV. The highest mean sample value for three different dilutions was 0.02 OD units. Of a total of 93 samples submitted for antigen typing, 80 (86%) tested positive by ELISA and 13 (14%) were negative. No FMDV was detected in ELISA-negative samples following attempted tissue-culture virus isolation.

KEYWORDS: ELISA – Foot and mouth disease – Thailand – Virus typing.

INTRODUCTION

Foot and mouth disease (FMD) is one of the most economically significant of all viral diseases of livestock. Three of the seven FMD virus (FMDV) serotypes (O, A and Asia 1) are endemic in Thailand, although these are restricted to central, northern and north-eastern areas of the country (12). FMD control in Thailand has depended on strategic use of monovalent vaccines (14), and reliable typing of outbreak virus has therefore been a necessary adjunct to disease control. The enzyme-linked immunosorbent assay (ELISA) is now a recommended test for detection of FMDV and typing of isolates (16). Westbury et al. (21) validated the ELISA for the diagnosis of FMD in Thailand at the Northern Veterinary Research and Diagnostic Centre (NVRDC) in Lampang, Thailand, using reagents produced at the Institute of Animal Health in Pirbright, United Kingdom (18). Subsequently, selected field isolates were


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chosen for the preparation of reagents suitable for FMD diagnosis in Thailand using ELISA. In applying these reagents to routine laboratory diagnosis of FMD at the NVRDC, it was necessary to confirm the established positive/negative threshold and examine the influence of heterotypic responses in the interpretation of results.

**MATERIALS AND METHODS**

**Indirect sandwich typing ELISA**

The ELISA method used here is similar to those described previously (9, 18, 21), with the following modifications: only types O, A and Asia 1 were involved in routine diagnosis, and the substrate used was tetramethylbenzidine (5). Reagents were produced at the Foot and Mouth Disease Centre in Pak Chong, Nakhon Ratchasima province (Thailand), as part of the Thai-Australian Foot and Mouth Disease Project. Field virus isolates and a single vaccine strain (O1NPT/65) were used as immunogens to produce trapping antisera in rabbits and detection antisera in guinea-pigs, as previously described (18). Details of virus isolates are given in Table I. Antisera in routine use were stored in glycerol (50% v/v) at −20°C. Standard control antigens were prepared and stored as described by Blacksell et al. (4). The standard control antigens and samples were arranged in a ‘four by four’ block pattern (4). Each sample was tested at three dilutions (undiluted, 1:2 and 1:4) and the corrected optical density (OD) results were calculated (see 18). Data analysis was simplified by the use of a mean sample value (MSV), defined as the mean of the corrected ODs of the three sample dilutions in each serotype system. A combination of antisera to FMDV type A (Table I) was used to ensure sensitivity to the range of type A field strains present in Thailand (13). A combination of reagents was not required for types O and Asia 1 because of the relative antigenic homology amongst field isolates of these serotypes (3, 7).

**Source and processing of submissions for routine FMDV typing**

The samples, which originated exclusively from regions five and six in northern Thailand (15), were collected between May and November 1990, and were transported to the NVRDC in phosphate-buffered glycerol (50% v/v glycerol, 50% v/v 0.04M

<table>
<thead>
<tr>
<th>Typing system</th>
<th>Rabbit antiserum</th>
<th>Guinea-pig antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>O₁HC547</td>
<td>O₁38/87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>O₁NPT/65 (a,b)</td>
</tr>
<tr>
<td>A (c)</td>
<td>A₂₂179/86</td>
<td>A₂₂179/86</td>
</tr>
<tr>
<td></td>
<td>A₁₅186/86 (a)</td>
<td>A₁₅186/86 (a)</td>
</tr>
<tr>
<td>Asia 1</td>
<td>Asia 1 45/88</td>
<td>Asia 1 36-2/88</td>
</tr>
</tbody>
</table>

(a) antisera pooled 1:2
(b) vaccine strain
(c) sub-types A₂₂ and A₁₅ as designated by Lunt et al. (13)
phosphate, pH 7.6). A 10% suspension of the epithelial tissue was prepared and tested by ELISA as previously described (18). Samples yielding a negative result in the ELISA were inoculated into primary or secondary bovine (calf) kidney (BK) cell-culture monolayers (2, 20). After three passages without the presence of cytopathic effect, the BK tissue-culture supernatants were tested for FMDV antigen using the ELISA.

Source and processing of tissues collected from FMD-free animals

A selection of 32 tongue epithelium samples was obtained from healthy cattle and buffalo during three visits to a local market. In addition, 18 interdigital samples (from cattle and pigs) and six tongue epithelium samples (from pigs) were collected from animals not exhibiting clinical symptoms of FMD which had been submitted to the NVRDC for post-mortem examination. Samples were processed in the same manner as submissions for FMDV typing.

RESULTS

Routine FMDV typing

From a total of 93 samples submitted, 80 (86%) gave positive reactions for FMDV and 13 (14%) tested negative. Of the 77 outbreaks investigated, 70 (87%) were confirmed by ELISA. Results for each serotype, indicating source of tissue, are summarised in Table II. An epizootic of type Asia 1 occurred in northern Thailand during the period of the study, which accounts for the predominance of Asia 1 typings.

TABLE II

Summary of foot and mouth disease virus typing of routine sample submissions tested by the indirect sandwich enzyme-linked immunosorbent assay (ELISA)

All samples testing negative by ELISA were inoculated onto bovine kidney cells for virus isolation

<table>
<thead>
<tr>
<th>Source of tissue</th>
<th>No. of samples tested</th>
<th>No. of samples positive in each typing system</th>
<th>No. of samples negative by ELISA and virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O</td>
<td>A</td>
<td>Asia 1</td>
</tr>
<tr>
<td>Cattle tongue</td>
<td>57</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Cattle foot</td>
<td>11</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Cattle heart</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cattle epiglottis</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cattle (unspecified)</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Buffalo tongue</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Buffalo (unspecified)</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pig tongue</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pig foot</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Pig snout</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Goat tongue</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>93</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>
All ELISA-negative samples also tested negative for FMDV isolation in BK cell cultures. The distribution of MSVs for each serotype (all samples) is presented in Figure 1. The evaluation of routine sample submissions yielding positive results indicated 91% with an MSV greater than 0.50, and 9% with an MSV between 0.20 and 0.50. The majority of samples in the lower range were from older, healing lesions or of sub-optimal quality. The highest MSV for a routine sample submission testing negative was 0.05.

**Tissue samples from FMD-free animals**

The following tissue samples from FMD-free animals gave MSV less than 0.02: buffalo tongue (8 samples), cattle tongue (24 samples), pig tongue (8 samples), cattle interdigital epithelium (12 samples), pig interdigital epithelium (6 samples).

![Figure 1](image)

**MSV:** mean of optical density readings for three different dilutions (undiluted, 1:2 and 1:4)

**FIG. 1**

*Distribution of mean sample values (MSVs) of optical density measured by indirect sandwich enzyme-linked immunosorbent assay for the detection and typing of foot and mouth disease virus in 93 field samples*
Heterotypic cross-reaction

Some samples yielded heterotypic responses (i.e. MSV greater than 0.10) in the typing systems for both type O and type Asia 1. A ratio greater than 0.33 for the MSV in the type O system relative to the type Asia 1 MSV was arbitrarily chosen as the level for significant inter-serotype cross-reactivity. The number of type Asia 1 positive samples showing significant levels of cross-reactivity in the type O system is shown in Table III. A total of five type Asia 1 positive samples (7.1% of total) gave levels of significant cross-reactivity in the type O system. However, as the type Asia 1 MSV increased (i.e. increasing levels of antigen), no increase in the level of significant cross-reactivity in the type O system was observed. On no occasion did the level of cross-reactivity prevent a definitive typing, and the cross-reactivity ratio never exceeded 0.45. Significant heterotypic cross-reactions were not observed in the types A and Asia 1 systems.

<table>
<thead>
<tr>
<th>Type Asia 1 MSV range</th>
<th>No. of type Asia 1 positive samples in MSV range</th>
<th>No. of significant cross-reactions *</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10-0.50</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>0.51-1.00</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>1.01-1.50</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>1.51-2.00</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>&gt;2.00</td>
<td>30</td>
<td>2</td>
</tr>
</tbody>
</table>

* A ratio of >0.33 for sample reactivity in the type O typing system relative to reactivity in the type Asia 1 typing system was arbitrarily determined as representing a significant cross-reaction

DISCUSSION

The ELISA was performed using reagents prepared against strains of FMDV endemic in Thailand. The relatively high percentage of typings for tissue samples suspected of being positive for FMDV was comparable to results from other studies using the indirect sandwich typing ELISA (9, 18, 21) and suggested an acceptable level of test performance.

The choice of MSV as the test variable enabled data reduction, while the advantages of serial dilution of the sample were retained. Dilution of the sample provided an indication of the quantity of antigen in relation to the control antigen dose/response curve. Samples yielding negative results in routine ELISA testing, and tissue samples from the animals clinically free from FMD which were tested in this study, gave MSVs less than 0.10. The overall distribution of results for routine sample submission reinforced the established positive/negative threshold of 0.10 OD (18) or, in the case of this study, 0.10 MSV. Samples which yielded positive typing results close to 0.10 MSV were generally from older, healing lesions (results not shown) of a type reported elsewhere as yielding lower results (17). The cells available for virus isolation were primary or secondary BK cell cultures, and virus isolation was attempted only on samples giving negative ELISA results. While BK cell cultures have been shown to be
useful for FMDV isolation (10), these are less sensitive than calf thyroid cultures (19, 20).
The sharp division in MSV between ELISA-positive and ELISA-negative samples, together with the failure to isolate FMDV in BK cells from ELISA-negative samples, suggested that the ELISA was almost as sensitive as virus isolation in BK cells, at least for the detection of serotype Asia 1.

Heterotypic cross-reactions were observed only in the type O system among samples positive for type Asia 1. However, this was always at a low MSV which did not prevent virus typing. Heterotypic reactivity of the antisera may be reagent- or sample-based (8). Different sera may vary in the degree of heterotypic reactivity – dependent on the method of preparation, the virus selected and the response of the animal – while samples may have different levels of 12S antigen, which may influence the level of cross-reactivity observed (1, 18). Transport of the samples to the NVRDC generally took four days, often at ambient temperature. Variability in the thermal stability of FMDV serotypes was noted by Doel and Baccarini (6), and extended exposure of samples to high ambient temperature may therefore increase the percentage of 12S sub-unit antigen, due to the breakdown of 146S antigen, possibly leading to a degree of heterotypic cross-reaction. In order to minimise antigen degradation in transit, adherence to recommended collection and transport procedures is advised (11).

ACKNOWLEDGEMENTS

The authors would like to thank Dr Ab Kongthon of the Department of Livestock Development, Foot and Mouth Disease Centre in Pak Chong, Nakhon Ratchasima province, Thailand, for providing the reference viruses used in this study. This study was funded by the Department of Livestock Development of the Royal Thai Government, the Australian Centre for International Agricultural Research and the Commonwealth Scientific and Industrial Research Organisation, Australian Animal Health Laboratory.

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Résumé : Un laboratoire central de Thaïlande a mis au point des antisérums contre les sérotypes endémiques (O, A et Asia 1) du virus de la fièvre aphteuse. Un laboratoire vétérinaire régional a ensuite utilisé ces antisérums dans un test immuno-enzymatique (enzyme-linked immunosorbent assay : ELISA) « sandwich » indirect, appliqué à la détection et au sérotypage de l'antigène du virus de la fièvre aphteuse. Lorsque le test ELISA présentait une densité optique inférieure à 0,10 unités, il était considéré comme négatif. Ce résultat a été confirmé à l'aide d'une cinquantaine de prélèvements de tissus reconnus exempts du virus de la fièvre aphteuse. La valeur moyenne supérieure de densité optique, avec trois dilutions différentes, était en effet de 0,02 unités. Sur un total de 93 prélèvements soumis au typage des antigènes, 80 (86 %) ont réagi positivement à l'épreuve ELISA et 13 (14 %) ont donné des résultats négatifs.
Le virus de la fièvre aphteuse n’a été décelé dans aucun des prélèvements ayant réagi négativement à l’épreuve ELISA, lors d’essais d’isolement en culture cellulaire.

**MOTS-CLÉS :** ELISA – Fièvre aphteuse – Thaïlande – Typage du virus.

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**Resumen:** Un laboratorio central de Tailandia produjo antisueros contra los serotipos del virus de la fiebre aftosa (O, A y Asia 1) endémicos en el país. Posteriormente, un laboratorio veterinario regional usó estos antisueros en una prueba inmunoenzimática (enzyme-linked immunosorbent assay: ELISA) «sandwich» indirecta aplicada a la detección y el serotipaje del antígeno del virus de la fiebre aftosa. Cuando la prueba ELISA presentaba una densidad óptica inferior a 0,10 unidades, su reacción se consideraba negativa, lo que se confirmó luego al utilizar 50 muestras de tejidos reconocidamente exentos de la enfermedad. El valor medio superior de densidad óptica, con tres diluciones diferentes, era, en efecto, de 0,02 unidades. De un total de 93 muestras de tejidos que se sometieron al tipaje de antígenos, 80 (86%) reaccionaron positivamente a la prueba ELISA y 13 (14%) en forma negativa. En ensayos de aislamiento en cultivos celulares, el virus de la fiebre aftosa no fue detectado en ninguna muestra que hubiera reaccionado negativamente a la prueba ELISA.

**PALABRAS CLAVE:** ELISA – Fiebre aftosa – Tailandia – Tipaje del virus.

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**REFERENCES**


