Use of combined Shewhart-CUSUM control charts in internal quality control of enzyme-linked immunosorbent assays for the typing of foot and mouth disease virus antigen

S.D. BLACKSELL *, L.J. GLEESON *, R.A. LUNT * and CHANPEN CHAMNANPOOD **

Summary: An enzyme-linked immunosorbent assay (ELISA) for the typing of foot and mouth disease virus (FMDV) antigen was employed for the routine laboratory diagnosis of FMD at a regional veterinary laboratory in northern Thailand. An objective procedure was developed to monitor the test performance of the ELISA, using absolute test control limits in a Shewhart-CUSUM (cumulative sum) control chart method. The procedure detected significant data trends and 'beyond control limit' situations for each antigen typing system (types O, A and Asia 1), using an assay variable (yi). Retrospective analysis using Shewhart-CUSUM control charts of data from 42 ELISAs demonstrated that control limits were exceeded in two assays for FMDV type A. The Shewhart-CUSUM control chart is a simple and reliable internal quality control method for the detection of significant random and systematic variation in assays.

KEYWORDS: ELISA – Foot and mouth disease – Quality control – Shewhart-CUSUM control charts.

INTRODUCTION

Detection and serotyping of foot and mouth disease virus (FMDV) antigen in field samples can be performed rapidly and with high sensitivity in the laboratory using an enzyme-linked immunosorbent assay (ELISA) (9). With minor modifications, this ELISA has been used for routine FMD diagnosis in a variety of situations (5,11), and may comprise up to seven separate antigen detection assays (for FMDV serotypes O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3) to be performed on a single test sample. The inherently complex nature of the ELISA can lead to results containing random error (e.g. reagent dilution error in a single test) or systematic variation (17) (e.g. gradual loss of assay sensitivity caused by peroxidase lability), and therefore periodic standardisation of reagents and internal quality control to monitor assay performance are desirable.


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Procedures for internal quality control of immunoassays have been investigated previously (7, 8, 14, 15, 16), but application of these procedures in the laboratory has been limited by the intrinsic problems of complex statistical processes and the multiplicity of assay designs. In this paper, the authors propose a simple internal quality control procedure for the ELISA, using the Shewhart-CUSUM (cumulative sum) control chart method, as described by Westgard et al. (13). A single reference variable was selected to reflect the performance of each antigen detection system in the ELISA, together with control limits which enable monitoring for both systematic and random assay variation. ELISA results from a regional veterinary laboratory in northern Thailand were retrospectively applied to the Shewhart-CUSUM control chart to demonstrate the usefulness of this approach for assay control.

Table I provides an explanation of the symbols and abbreviations used in this method.

<table>
<thead>
<tr>
<th>Symbol/abbreviation</th>
<th>Explanation</th>
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<tbody>
<tr>
<td>$t$</td>
<td>Process mean</td>
</tr>
<tr>
<td>$s$</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>$y_i$</td>
<td>Test variable used to monitor test performance</td>
</tr>
<tr>
<td>$k$</td>
<td>Warning limit set at $\pm 1s$ for CUSUM initiation</td>
</tr>
<tr>
<td>$h$</td>
<td>Defective test limit set at $\pm 2.7s$. When $y_i$ exceeds the $h$ limit, the test is considered defective</td>
</tr>
<tr>
<td>$d_i$</td>
<td>Used to calculate CUSUM value. The difference between $y_i$ and the $k$ limit</td>
</tr>
<tr>
<td>$OD_n$</td>
<td>Optical density of standard control antigen dilution</td>
</tr>
<tr>
<td>CUSUM</td>
<td>$\Sigma d = d_1 + d_2 + d_3 + d_4 \ldots$</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**ELISA procedure**

The indirect sandwich typing ELISA (9) was performed with the following modifications: antigen detection systems were used only for types O, A and Asia 1; and tetramethylbenzidine was used as the substrate (4). Briefly, the modified ELISA involved testing up to five samples in a 'four by four' block pattern, together with control antigens for each antigen detection system (Fig. 1). In the control antigen block, the three twofold dilutions of each serotype were placed in the first three wells of the appropriate row. Sample dilutions were similarly added to each serotype detection system in the test blocks. The last well in each row of each block served as the background control well for the serotype. The mean optical density (OD) of the background control wells for each serotype detection system was calculated for the plate and subtracted from all OD readings of the corresponding detection system to give the corrected OD used for analysis of results from the test (3) and control samples. This paper is concerned only with the use of the results from control antigen wells (not from test antigen wells) in an assessment of assay performance.
Control antigens for types O, A, and Asia 1 were produced from tissue-culture supernatant fluids harvested from baby hamster kidney (BHK) monolayers infected with the following vaccine challenge virus types: O₁ Bangkok 1960 (O/BKK/60), A₁₅ Bangkok 1960 (A/BKK/60) and Asia 1 Bangkok 1960 (Asia 1/BKK/60). Foetal calf serum was added (25% v/v) to control antigens which were stored in glycerol (50% v/v) at -20°C.

The performance of each antigen detection system was assessed as the mean OD for the detection of homologous control antigen at three dilutions. The corrected OD values (ODₚ, OD₪ and ODᵣ) for the three dilutions (p, q and r) of each control antigen were used to calculate the quality control variable ($γ_i$) for the ith test, as follows:

$$γ_i = \frac{(OD_p + OD_q + OD_r)}{3}.$$
For this value to provide the most reliable reflection of assay performance, the dilution levels were determined by pre-titration to fall within the linear region of the dose/response curve (Fig. 2). The three dilution levels were then fixed for all subsequent ELISA testing of the control antigens. Linear regression analysis was performed following each ELISA to assess the relationship between antigen input at the three control antigen dilution levels and the corresponding OD values. A coefficient of determination of less than 0.95 was regarded as indicating unacceptable deviation from the expected linearity.

**FIG. 2**

Dose/response curve of foot and mouth disease virus control antigen type O (+) measured by indirect sandwich typing enzyme-linked immunosorbent assay, indicating the approximately linear portion and the three control antigen dilutions (p, q and r) chosen for Shewhart-CUSUM analysis

**Use of \( \gamma_i \) in Shewhart-CUSUM control charts**

The reference variables (\( \gamma_i \)) for each serotype (O, A or Asia 1) were used to monitor assay performance based on a method described previously (13). In this method, two quality control charts were used: the Shewhart control chart, which held the plot of the \( \gamma_i \) value, and the CUSUM chart showing a trend analysis of the \( \gamma_i \) value. These charts were combined to form a single graph with a dual y axis (Fig. 3).
Sample Shewhart-CUSUM control chart indicating CUSUM points (●) and $\gamma_i$ (●)

See Table I for an explanation of symbols.

Tests 1 to 3 fell within the $k$ limits. Test 4 exceeded the upper $k$ limit and CUSUM was initiated (i.e. $d_1 = \gamma_4 - k_{upper}$). The CUSUM calculation continued for tests 5 to 7 (i.e. $\Sigma d_7$) until test 7, when the CUSUM value crossed the zero line and CUSUM calculation ceased. Tests 8 and 9 fell within the $k$ limits. Test 10 exceeded the lower $k$ limit and CUSUM was initiated. $d_{l_1}$ was calculated for test 10 and the CUSUM value was calculated (i.e. $d_{l_1} = \gamma_{10} - k_{lower}$). The CUSUM calculation continued for tests 11 to 13, when the CUSUM value exceeded the lower $h$ limit, indicating an unacceptable data trend.

This chart was used to establish the following data:

- the proximity of the $\gamma_i$ value to a target mean ($t$)
- whether this represented an acceptable result
- whether any undesirable trend was indicated.

The target mean ($t$) was based on the mean $\gamma_i$ values from at least fifteen tests (13). Analysis was facilitated by the establishment of upper and lower limits on either side of the target mean ($t$), as follows:

- a warning limit ($k$) at which the CUSUM trend analysis was initiated
- a defective test limit ($h$) which, if exceeded by either the CUSUM plot or the $\gamma_i$ value plot, provided an indication of unacceptable assay performance.
RESULTS

Selection and suitability of $\gamma_i$ as a reference variable

The results of 42 ELISA tests on FMDV samples, performed over a seven-month period at the Northern Veterinary Research and Diagnostic Centre (NVRDC), Hang Chat, Lampang, Thailand, were used for the formulation of quality control procedures. Tests were performed by three different operators over the seven-month period. The linearity of the OD value as a function of antigen dilution for each of the control antigens (O, A and Asia 1) was examined, and all tests demonstrated linearity (results not shown) of these functions, thus indicating acceptability of the $\text{OD}_p$, $\text{OD}_q$ and $\text{OD}_r$ results for the calculation of $\gamma_i$ values.

Using levels suggested by Westgard et al. (13), the upper and lower warning limits ($k_{\text{upper}}$ and $k_{\text{lower}}$) were set at $\pm 1s$ from the zero CUSUM line, and the upper and lower defective test limits ($h_{\text{upper}}$ and $h_{\text{lower}}$) were set at $\pm 2.7s$ ($s$: standard deviation).

The Shewhart-CUSUM control chart functioned in two modes. When the $\gamma_i$ value was within the warning limits ($k_i$), the $\gamma_i$ value alone was plotted on the chart (using the OD axis on the left). If a large test error caused $\gamma_i$ value to fall beyond the defective test limits ($h_i$), the test performance was considered defective. Alternatively, if the $\gamma_i$ value was outside one of the $k$ limits, but within the $h$ limits, the CUSUM trend analysis would commence. The CUSUM value ($\Sigma d_i$) was calculated as the cumulative sum of the differences between the CUSUM-initiating $\gamma_i$ value (and subsequent $\gamma_i$ values) and the level of the exceeded warning limit ($k$) (13). For example (Fig. 3), if the $\gamma_i$ value exceeded the upper $k$ limit ($k_{\text{upper}}$) in the 4th test in a series ($i = 4$), the CUSUM calculation for this and subsequent tests might be represented as follows:

\[
\begin{align*}
\text{4th test} & \quad \Sigma d_4 = d_1 = (\gamma_4 - k_{\text{upper}}) \\
\text{5th test} & \quad \Sigma d_5 = d_1 + d_2 = (\gamma_4 - k_{\text{upper}}) + (\gamma_5 - k_{\text{upper}}) \\
\text{6th test} & \quad \Sigma d_6 = d_1 + d_2 + d_3 = (\gamma_4 - k_{\text{upper}}) + (\gamma_5 - k_{\text{upper}}) + (\gamma_6 - k_{\text{upper}}) \\
\text{ith test} & \quad \Sigma d_i = d_1 + d_2 + d_3 + ... + d_{(i-3)}. \end{align*}
\]

The CUSUM value ($\Sigma d_i$) was plotted on the Shewhart-CUSUM chart (using the CUSUM axis on the right). A line representing the zero CUSUM level was fixed at the target mean level ($t$), and the scale of the CUSUM axis ranged from $-2.7s$ to $+2.7s$ on either side of this line. The CUSUM trend analysis continued until the plot was observed to cross either the defective test limit ($h$) or the zero CUSUM line. If the $h$ limit was crossed by the CUSUM plot, the test performance would be considered to be defective. However, if the zero CUSUM line was crossed by the CUSUM plot, the chart would revert to a single mode (Shewhart plot) in which only the $\gamma_i$ value was plotted.

Summary statistics

Summary statistics of $\gamma_i$ populations were calculated using the statistical software package MINITAB version 7. Normal distribution of $\gamma_i$ populations was assessed using acceptance criteria applied to the Wilk-Shapiro/Rankit plot test for normality (10). Randomness of the populations was tested using the Runs test, at a significance of $P = 0.050$.
The $\gamma_i$ value was dependent on a data reduction of the linear function used to describe the relationship between OD$_p$, OD$_q$, and OD$_r$, and the corresponding antigen dilutions. Independent variation in the slope parameter ($a$) could potentially be a component of variability not represented in the $\gamma_i$ value. Linear regression analysis was performed on the slope ($a$) and intercept ($b$) parameters generated from all testing of control antigen dilutions. A positive correlation ($r = 0.867$) was observed between the $a$ and $b$ parameters, suggesting that control antigen data may be summarised by selecting a single variable ($\gamma_i$) for use in quality control analysis.

**Application of $\gamma_i$ data to Shewhart-CUSUM control charts**

The target means ($t$) and associated standard deviation values ($s$) were determined retrospectively using 15 $\gamma_i$ values (separately for O, A and Asia 1) randomly selected from the results of 42 tests. Summary statistics and CUSUM target values calculated from the fifteen randomly selected $\gamma_i$ values for each serotype are presented in Table II. The values for $\gamma_{1-42}O$, $\gamma_{1-42}A$, and $\gamma_{1-42}Asia$ 1 exhibited random distributions. Shewhart-CUSUM control charts were plotted retrospectively using these $\gamma_i$ values (Figures 4a, 4b and 4c). The satisfactory performance of the antigen capture systems for types O and Asia 1 was indicated by the fact that all $\gamma_{1-42}O$ and $\gamma_{1-42}Asia$ 1 values fell within the $h$ limits, and by the absence of persistent undesirable trends (i.e. progressing beyond the $h$ limits) in the CUSUM plot. The $\gamma_i$ value for the type A control antigen in test no. 18 (i.e. $\gamma_{18}A$) gave a value beyond the lower $h$ limit (i.e. $t < -2.7s$), indicating unacceptable random variation. Similarly $\gamma_{29}A$ gave a $t$ value greater than $+2.7s$, also indicating unacceptable random variation. These single values indicated large, random variation which was attributed to reagent dilution error, and the tests were repeated. While CUSUM plots were initiated for the three antigen typing systems, in no case did trends persist to the point of test rejection (i.e. beyond the $h$ limit). Also noted was the tendency for CUSUM plots to be initiated simultaneously for more than one antigen detection system.

**Table II**

*Summary statistics and CUSUM target values calculated from fifteen randomly-selected $\gamma_i$ values derived from each of the $\gamma_{1-42}O$, $\gamma_{1-42}A$ and $\gamma_{1-42}Asia$ 1 populations*

<table>
<thead>
<tr>
<th>Parameter *</th>
<th>O</th>
<th>A</th>
<th>Asia 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t$</td>
<td>0.941</td>
<td>0.878</td>
<td>0.977</td>
</tr>
<tr>
<td>$s$</td>
<td>0.204</td>
<td>0.207</td>
<td>0.359</td>
</tr>
<tr>
<td>$k_{\text{lower}}$</td>
<td>0.737</td>
<td>0.671</td>
<td>0.626</td>
</tr>
<tr>
<td>$k_{\text{upper}}$</td>
<td>1.145</td>
<td>1.085</td>
<td>1.336</td>
</tr>
<tr>
<td>$h_{\text{lower}}$</td>
<td>0.390</td>
<td>0.319</td>
<td>0.008</td>
</tr>
<tr>
<td>$h_{\text{upper}}$</td>
<td>1.492</td>
<td>1.437</td>
<td>1.946</td>
</tr>
<tr>
<td>Wilk-Shapiro **</td>
<td>0.945</td>
<td>0.945</td>
<td>0.956</td>
</tr>
<tr>
<td>Runs test ($P$) ***</td>
<td>0.894</td>
<td>0.970</td>
<td>0.999</td>
</tr>
</tbody>
</table>

* See Table I for an explanation of symbols
** Wilk-Shapiro values approaching 1.000 indicate population normality
*** Runs test is significant at $P < 0.050$, therefore $P$ values greater than 0.050 indicate population randomness
FIG. 4a

Shewhart-CUSUM control chart of $\gamma_{1-42O}$, indicating CUSUM points (●) and $\gamma_i$ (●)

See Table I for an explanation of symbols

DISCUSSION

One of the principal difficulties for the application of quality control procedures to a routine ELISA is the definition of a single reference variable which adequately describes significant changes in test performance while not being excessively sensitive to inherent ELISA variability. The $\gamma_i$ value effectively summarised information from the control antigen titration curve while reflecting changes in the two variables ($a$ and $b$) which may influence this curve.

Major advantages of Shewhart-CUSUM control charts are the ease of application, and interpretation by the use of absolute CUSUM limits. Reports of previous applications of CUSUM methods to immunoassays have described the use of the 'V-mask' method of quantitative interpretation (2, 7, 15, 16). Westgard et al. (13) suggested that the use of absolute CUSUM limits provided a valid alternative to V-mask, with a simplified decision process. When applied to a diagnostic laboratory, CUSUM limits must be set by first establishing a target mean ($t$) and standard deviation ($s$) using $\gamma_i$ data from at least fifteen tests. CUSUM control rules are then
chance to achieve the most rapid detection of an undesirable trend in the \( \gamma_1 \) variable (i.e. probability of error detection: \( p_{\text{ed}} \)) while minimising the likelihood of a randomly distributed set of results being incorrectly identified as an undesirable trend (i.e. a low probability of false rejection: \( p_{\text{fr}} \)). A probability of false rejection in the range of 0.01 to 0.05 is considered to be acceptable for a clinical laboratory (12). The CUSUM rule with the \( k \) limits set at ±1.0s and \( h \) limits at ±2.7s allows for a \( p_{\text{fr}} \) of approximately 0.002, while maintaining a relatively high probability for detection of systematic drift, as well as monitoring the level of random error (13). Incorporation of the Shewhart control limits at \( k = \pm 1.27s \) provides a combination with improved \( p_{\text{ed}} \) for both systematic and random errors (13). Westgard et al. (13) also suggested that familiarity with the performance characteristics of an assay would enable the establishment of more stringent \( k \) and \( h \) limits.

In the development of this quality control method, \( \gamma_1 \) data for each serotype could not be assessed independently of two factors: the coefficient of determination \( (r^2) \) for the relationship between \( \text{OD}_{p}, \text{OD}_{q}, \text{OD}_{r} \) and control antigen dilution; and statistical evaluation of normal and random distribution. Values of \( r^2 \) lower than 0.95 would have
FIG. 4c

Shewhart-CUSUM control chart of $\gamma_{1-42} \text{Asia 1}$, indicating CUSUM points (•) and $\gamma_t$ (○)

See Table I for an explanation of symbols

indicated that $\gamma_t$ was not representing the linear portion of the dose/response curve, and so the assumptions supporting the use of this value in Shewhart-CUSUM control charts would not have been valid. Furthermore, the assumptions made in the use of control charts are that the variation of $\gamma_t$ is independent between runs, and normally distributed. Deviations from these assumptions can produce misleading results (1). The $\gamma_t$ populations for all serotypes were in accordance with the required statistical assumptions.

An objective quality control procedure can serve several purposes when applied to indirect sandwich ELISA for typing FMDV in diagnostic laboratories. Such a procedure provides a historical record of test performance (useful to both operators and field investigators), assures consistency of performance between laboratory sites, and indicates whether errors are random or systematic. When quality control criteria are met, negative results or apparent decreased sensitivity of the test on good quality samples could alert laboratory personnel to significant endemic strain variation, or the incursion of a new serotype or sub-type. The quality control method described above is simple, and readily applicable to a regional diagnostic laboratory without a requirement for elaborate equipment. Production of standardised control antigen reagents is simple and
inexpensive, and these reagents are extremely stable when stored at -20°C (6). The combination of standardised reagents in kit form and an appropriate quality control method could improve the performance and uniformity of laboratory diagnosis of FMD.

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Résumé : Un laboratoire régional du nord de la Thaïlande a appliqué l'épreuve immuno-enzymatique (enzyme-linked immunosorbent assay : ELISA) en vue du typage de l'antigène du virus de la fièvre aphteuse dans le cadre du diagnostic de routine de cette maladie. Un protocole objectif a été mis au point pour contrôler les performances de l'épreuve ELISA, qui utilise les limites absolues de confiance selon la méthode de graphique de contrôle « Shewhart-CUSUM » (CUSUM : cumulative sum : somme cumulée). Cette procédure a permis d'observer d'importants écarts et des cas où les limites de confiance étaient dépassées pour chaque système de typage d'antigène (types O, A et Asia 1) en utilisant une variable (y). Une analyse rétrospective, basée sur les graphiques de contrôle « Shewhart-CUSUM » des données relatives à 42 épreuves ELISA, a démontré que les limites de confiance étaient dépassées pour deux d'entre elles (type A du virus de la fièvre aphteuse). Le graphique de contrôle « Shewhart-CUSUM » est une méthode de contrôle de qualité interne à la fois simple et fiable permettant de décider d'importants écarts aléatoires et systématiques dans les épreuves.

MOTS-CLÉS : Contrôle de qualité – ELISA – Fièvre aphteuse – Graphiques de contrôle « Shewhart-CUSUM ».
Resumen: Un laboratorio regional del norte de Tailandia aplicó la prueba inmunoenzimática (enzyme-linked immunosorbent assay: ELISA) para el tipaje del antígeno del virus de la fiebre aftosa como parte del diagnóstico de rutina de esta enfermedad y, para controlar los resultados de la prueba, estableció un protocolo objetivo que utiliza los límites absolutos de confianza según la gráfica de control «Shewhart-CUSUM» (CUSUM: cumulative sum, suma acumulada). Este procedimiento permitió observar diferencias significativas y casos en que se excedían los límites de confianza para cada sistema de tipaje del antígeno (O, A y Asia 1) a partir de una variable (yi). Un análisis retrospectivo en que se utilizó las gráficas «Shewhart-CUSUM» de los datos de 42 pruebas ELISA mostró que se excedían los límites de confianza en dos de estas pruebas (tipo A del virus de la fiebre aftosa). La gráfica de control «Shewhart-CUSUM» es un método de control de calidad interno sencillo y fiable que permite detectar importantes diferencias aleatorias y/o sistemáticas en las pruebas.

PALABRAS CLAVE: Control de calidad – ELISA – Fiebre aftosa – Gráfica de control «Shewhart-CUSUM».

REFERENCES


