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Uncertainty of measurement for competitive and indirect ELISAs

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Summary
A method for the estimation of the uncertainty of measurements for Gaussian outcomes of enzyme-linked immunosorbent assay (ELISA) is described using competitive and indirect foot and mouth disease (FMD) ELISAs. Assay repeatability was determined by random effects analysis of variance, and the normality of the residuals was checked. The standard errors of the individual predicted values were transformed into confidence intervals around the corresponding observed values and further transformed into probabilities of being above/below a cut-off. Logistic regression models were subsequently used to interpolate probability values for the whole range of possible assay values. The uncertainty of measurement of a test result was finally defined as the probability of not observing the same qualitative test result when retesting the same sample. For the competitive ELISA any sample with a percent inhibition 4% above the cut-off value had an uncertainty level (probability of a negative result in the case of retest) below 5%. In the indirect ELISA with a cut-off OD of 0.1, the uncertainty was below 5% for any sample with a normalised OD value above 0.22.

Keywords
Solid phase competition ELISA (SPCE) – Foot and mouth disease – Indirect ELISA – Logistic regression model – Repeatability – Uncertainty of measurement.

Introduction

Diagnostic laboratories are strongly encouraged to perform their tests within a quality control management system (1). Accreditation according to ISO/IEC 17025 (9), the standard for competence of testing and calibration laboratories, requires the set-up of a validation file containing information on:

– the intrinsic characteristics of the test (sensitivity, specificity, relative trueness, limit of detection/ quantification)

– repeatability and intermediate precision (agreement between replicates within and between runs of the assay, respectively, within a laboratory)

– reproducibility (the ability of a test method to provide consistent results when applied to aliquots of the same samples tested at different laboratories)

– the evaluation of the uncertainty of measurement (for microbiological laboratories: EA-04/10, 2002) (2).

The terms repeatability and reproducibility are in line with the definitions of the World Organisation for Animal

Health (OIE) (13). According to the International Laboratory Accreditation Cooperation (ILAC-G17) (8), uncertainty of measurement can be defined as 'a parameter associated with the result of a measurement that characterises the dispersion of the values that could reasonably be attributed to the measurand'; the measurand is 'a particular quantity subjected to measurement'. In practice, uncertainty of measurement has two aspects (9):

a) identification of all components that influence the uncertainty, which generally include laboratory equipment, environment (temperature), sample conditions, consumables, staff, and the procedure itself. Once the components have been identified, their individual influence on the test result has to be determined and, if necessary, action should be taken to keep critical components within acceptable limits (e.g. calibration of pipettes to ensure that correct volumes are dispensed);

b) quantification of the uncertainty itself, which should result in a quantitative figure accompanying each test result that can be sent to the client upon request.

Until now, guidelines for the estimation of the uncertainty of measurement have primarily focused on truly quantitative tests, which are commonly found in the field of chemistry (3). This approach is not readily applicable to diagnostic tests, in which the final result is usually binary (positive or negative – with an optional category for doubtful results). Therefore, the goal of the present study was to develop a methodology for the estimation of uncertainty of measurement for use with enzyme-linked immunosorbent assays (ELISAs), which are widely used in the diagnosis of infectious diseases including foot and mouth disease (FMD). The method was developed and validated using several datasets generated by competitive and indirect ELISAs for the detection of specific antibodies against FMD virus (FMDV) and FMDV antigens, respectively.

Materials and methods

Diagnostic tests evaluated

A modified version of the solid phase competition ELISA (SPCE) described and validated by Paiba *et al.* (11) is used as our in-house SPCE standard operating procedure (SOP) (6) for the detection of specific anti-FMD antibodies. Briefly, ELISA plates are coated with rabbit anti-FMDV type O polyclonal antiserum at a predetermined concentration for capturing FMD virus. The test serum, 1:10 pre-diluted in phosphate buffered saline containing Tween [AquMan] (PBST), is added at the same time as a predefined concentration of competing polyclonal guinea pig anti-FMDV type O antiserum and incubated at 37°C for one hour. After a wash step an optimal concentration of polyclonal rabbit anti-guinea pig immunoglobulins conjugated with horseradish peroxidase (HRPO) is added and incubated at 37°C for another hour. A final wash step is performed before the substrate is added for 15 min and the optical density (OD) is read at 490 nm. The result is expressed as percentage inhibition (PI) [(ODmax – ODsample)/ODmax]*100, where the ODmax is given by the no serum control. The protocol described in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (12) allows for a grey (doubtful) zone of interpretation between PIs of 40 and 60. Rather than using fixed PIs, the lower and upper cut-off values in our SPCE are deduced from the value observed for a cut-off sample (CO) that is incorporated on each test plate. This variable cut-off is used to compensate for the observed minor daily variations in PI. Samples with a PI < the lower cut-off (PI CO –4%) are scored negative, whereas samples with a PI > the upper cut-off (PI CO +15%) are considered positive.

An indirect ELISA (I-Ag-FMD) (4, 12) is used in our laboratory for the detection of FMD antigens. Very briefly, ELISA plates are coated with rabbit anti-FMDV type C polyclonal antiserum, prior to adding the FMDV test sample and incubation at 37°C for one hour. After a wash step, a predefined concentration of polyclonal guinea pig anti-FMDV type C antiserum is added to the wells and incubated at 37°C for one hour. After another wash step an optimal concentration of polyclonal rabbit anti-guinea pig immunoglobulins conjugated with HRPO is added and incubated at 37°C for a further hour. A final wash step is performed before addition of the substrate for 15 min and reading the OD at 490 nm. The I-Ag-FMD ELISA does not have a 'grey' zone, and samples are considered positive when the difference between their OD and the OD of a blank sample is greater than 0.1.

Both tests were manufactured in house by combining reagents prepared in house (FMDV, buffers) with reagents (rabbit and guinea pig anti-FMDV antisera) provided by the World Reference Laboratory for FMD (Institute for Animal Health, Pirbright, United Kingdom).

Samples used for evaluation

The uncertainty associated with the SPCE was assessed using serial two-fold dilutions of a strong anti-FMDV antibody positive serum directed against FMDV serotype O (6). The I-Ag-FMD ELISA was evaluated using serial two-fold dilutions of FMDV (strain C_1 Noville) cultivated on SK6 cells (10). The dilution series was designed to cover the entire detection range of the tests, from the upper plateau to the lower plateau. The dilutions were prepared and aliquoted on a single day to ensure homogeneity of the samples and to minimise any error associated with

preparation of the dilutions. Single-use aliquots of the serum dilutions were stored at –20°C; the virus samples were kept at –80°C until use. All dilutions were tested in duplicate on ten different days and by at least three different technicians.

Statistical methods

The repeatability and intermediate precision (within and between runs) of the assays were determined for each level of analyte (each level corresponded to one dilution) using random effects analysis of variance. They were expressed as coefficients of variation (CV; standard deviation of replicates divided by mean of replicates) using the 'NESTED' procedure in SAS statistical software (SAS Institute Inc.v9.2). Normality of the residuals was checked using the Shapiro-Wilk test (7). The standard errors of the individual predicted values were subsequently transformed into confidence intervals around the corresponding observed values and into probabilities of being above or below the cut-off value using the normal law (Fig. 1). Finally, logistic regression models were deployed to interpolate a probability value for any possible result that fell within the detection range of the test. The uncertainty in a positive (or negative) test result was defined as the probability of not observing the same qualitative test result (positive or negative) when retesting the same sample a second time.

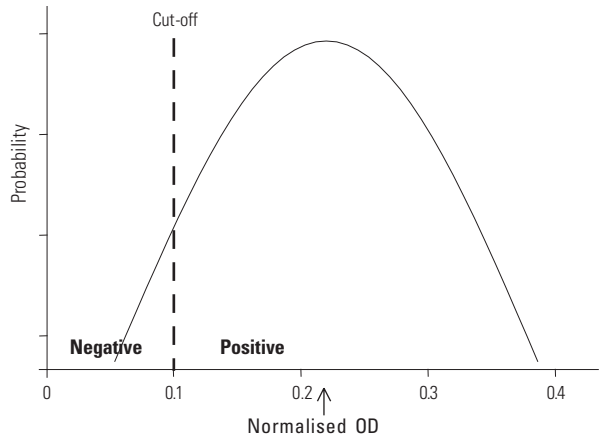
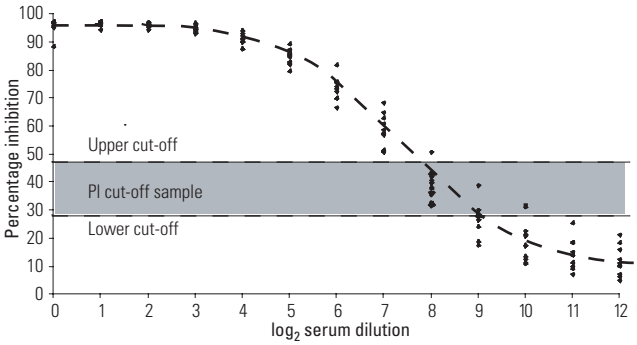


Fig. 1
Transformation of standard errors in probabilities using the normal law
For each observed value (y_{ij}), the predicted standard (d_{ij}) error was transformed into a probability using the normal distribution $N(y_{ij},(d\hat{}_{ij})^2)$ and the cut-off. The certainty of a decision (positive/negative) was then estimated as the proportion of the confidence distribution falling above/below the cut-off. The arrow represents one individual value y_{ij} that was taken as an example: sample with a normalised OD value of 0.22 having a 95% probability of positive retest result (certainty) and a 5% probability of negative retest result (uncertainty) (see also Results, Table II)

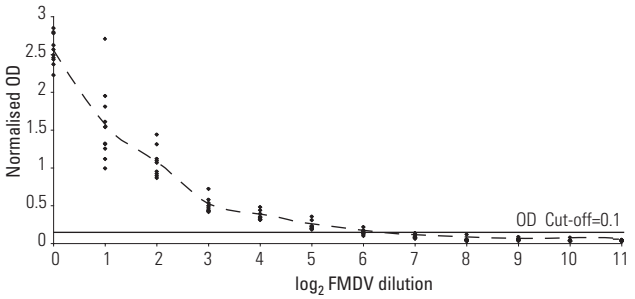
Because the SPCE for FMD uses a variable cut-off, the analysis and modelling were performed on the differences between the PI observed for each sample and the PI observed for the CO. For the indirect I-Ag-FMD ELISA, the analysis was performed on normalised OD values obtained by subtracting the OD of the blank sample (background) from the OD value of each sample.

Results

Analysis of the results obtained from the anti-FMDV serum by the SPCE on ten different days and from the FMDV dilutions by the I-Ag-FMD generated two datasets. The PI obtained for the serum dilutions completely covered the higher plateau and the linear phase of the ELISA, but only partly covered the lower plateau (Fig. 2a). In contrast, the OD values for the dilutions of the virus preparation adequately covered the linear phase and the lower plateau, but did not fully cover the higher plateau (Fig. 2b).



a) A competitive ELISA for the detection of FMDV-specific antibody (solid phase competition ELISA)
In this example the cut-off sample has a PI = 32 (variable cut-off). Samples with a PI < the lower cut-off (PI CO –4%) are scored negative, whereas samples with a PI > the upper cut-off (PI CO +15%) are considered positive



b) Indirect ELISA for the detection of the FMDV antigen

Fig. 2
Datasets used for the development and the validation of the model to determine the uncertainty of measurement
In both tests each level (dilution) is represented by ten results obtained on ten different days

The percentage CV for repeatability and intermediate precision (the latter reflects the highest level of variation within a single laboratory) reached 2.9% and 6.6% respectively for the SPCE, and 13.4% and 21.7% for the I-Ag-FMD ELISA.

In contrast to the I-Ag-FMD, for which the standard errors were normally distributed at all levels, the five levels corresponding to the higher concentrations of antibodies showed a non-normal distribution for the SPCE (Table I).

Table I
Normality test on the residuals from the analysis of variance

	Solid phase competition ELISA		Indirect ELISA	
Log_2 dilution[a]	p value	Conclusion	p value	Conclusion
0	0.010	Non normal	0.775	Normal
1	0.010	Non normal	0.970	Normal
2	0.010	Non normal	0.884	Normal
3	0.010	Non normal	0.402	Normal
4	0.021	Non normal	0.420	Normal
5	0.093	Normal	0.995	Normal
6	0.356	Normal	0.894	Normal
7	0.845	Normal	0.145	Normal
8	0.334	Normal	0.150	Normal
9	0.824	Normal	0.738	Normal
10	0.999	Normal	0.141	Normal
11	0.314	Normal	0.655	Normal
12	0.557	Normal		

a) Each dilution, corresponding to a level of analyte (antibody or virus), was tested in duplicate on ten separate days

Individual probabilities of scoring positive or negative were deduced from the standard errors of the individual predicted values, and the logistic model was used to interpolate the probabilities from the dataset to the entire range of possible test results. Figure 3 depicts the fit of the logistic model to the data from the I-Ag-FMD ELISA. The estimations generated for both tests are summarised in Table II, which shows that the PI (SPCE) and the normalised OD values (I-Ag-FMD) were associated with the reference probabilities of positive or negative test results. For example, every sample with a PI higher than 3.42% above the upper cut-off had a probability of at least 95% of showing a positive result in the case of a retest. Similarly, a sample with an observed PI 5.83% below the lower cut-off has a 95% probability of giving a negative result after a retest and, thus, an uncertainty of less than 5%. For the I-Ag-FMD ELISA, every sample with a normalised OD value of 0.19 had a 90% probability of a positive result on retest (certainty) and a 10% probability of a negative retest result (uncertainty).

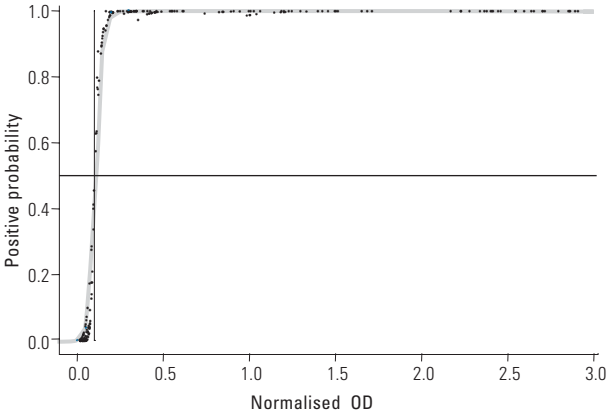


Fig. 3
Fit of the logistic model with the individual predictive positive probability values calculated for the dataset generated with the indirect ELISA

Table II
Results associated with the probabilities of positive/negative test results

p value	Solid phase competition ELISA		Indirect ELISA	
	Positive [a]	Negative	Positive [b]	Negative
0.99	5.35	–9.1	0.28	–0.04
0.95	3.42	–5.83	0.22	0.02
0.9	2.54	–4.36	0.19	0.04
0.7	0.96	–1.68	0.15	0.09
0.5	–0.03	0	0.12	0.12
0.3	–1.02	1.67	0.09	0.15
0.1	–2.61	4.35	0.04	0.19
0.05	–3.48	5.83	0.02	0.22

a) For the solid phase competition ELISA (SPCE), the model converts the difference between the result for the sample and the upper cut-off (or the lower cut-off for negative results) into a probability: e.g. a sample showing 5.35 PI above the upper cut-off has a 0.99 probability of a positive retest result and a sample with 4.36 PI below the lower cut-off has a 0.9 probability of a negative retest result

b) For the indirect ELISA, the model converts the normalised OD values (i.e. $OD_{test\ sample} - OD_{blank}$) into a probability (e.g. a sample with a normalised OD above 0.28 has a 0.99 probability of positive retest result)

Discussion

The estimation of the uncertainty of measurement is of growing concern to the authorities in charge of accrediting microbiological laboratories. However, there are currently no specific guidelines for the quantification of uncertainty of measurement for microbiological tests. This paper partly addresses this problem by describing a method that is suitable for one of the most widespread tests, the ELISA.

The first component of the evaluation of uncertainty relies on:

– the identification of the parameters that could potentially affect the uncertainty

– the identification of critical parameters by testing small, deliberately introduced, deviations

– the adoption of appropriate actions to maintain the critical parameters within an acceptable range.

The major factors to take into account are listed in the introduction. However, the critical factors can differ significantly depending on the method to be validated, and their identification may rely mainly on experimental testing. This first component of the evaluation of uncertainty is straightforward and is not the topic of the present paper, which primarily focuses on quantifying uncertainty of measurement.

Before proceeding with data analysis and quantification of uncertainty, it is necessary to generate a dataset comprising test results associated with some key probabilities (e.g. 5% or 1% uncertainty). For ELISAs, as a guide, we recommend the use of several two-fold dilutions of a strongly positive sample including:

– three dilutions covering the higher plateau

– several dilutions covering the linear phase

– three dilutions covering the lower plateau.

The examples presented here suggest that it is not always easy to chose appropriate dilutions (the strongly positive serum was not diluted sufficiently and, consequently, the lower plateau was not covered by three dilutions) or to obtain sufficiently concentrated reagents (the titre of the pure virus preparation was too low and the OD value of the second dilution was no longer part of the higher plateau). Even though these sample preparations did not comply completely with the above-mentioned recommendation, both datasets gave reliable results because the 95% and even the 99% certainty (5% and 1% uncertainty) could be estimated by the model and corresponded to test results that were covered by the dataset. Where possible, however, we advise that the whole range of data be covered, because the validity of a dataset can only accurately be assessed after completion of the analysis, and failure to validate a dataset requires the preparation and analysis of a new panel of samples.

The observed repeatability values for the tested ELISAs illustrate the difference between a test that is highly normalised (in our case the competitive ELISA) and a test with minor or no normalisation (the indirect ELISA). The SPCE results are normalised twice by calculation of the percentage inhibition as well as by use of a variable cut-off. With this test, the CVs for repeatability and intermediate precision were both below 10%. In contrast, the I-Ag-FMD results are only moderately normalised by subtracting the background OD from the sample OD, which resulted in higher CVs for repeatability and intermediate precision. Nevertheless, the overall CV of the latter test remained below 25 percent, which is considered to be the upper acceptable value for an ELISA (5). Additionally, the assays differed greatly in relation to uncertainty of measurement. For instance, a serum sample having a percentage inhibition slightly above the upper threshold was associated with a high probability of a positive retest result and a low uncertainty in the competitive ELISA, whereas a virus sample with a normalised OD value of 0.19 (i.e. nearly twice the cut-off) is only associated with a 90% probability of scoring positive if retested.

Despite its higher level of uncertainty, the I-Ag-FMD can still be assumed to be fit for purpose because its use is mostly restricted to two particular situations:

a) the confirmation of a positive cytopathogenic effect in cell culture, in which case the associated OD values usually exceed 0.6 even when a single small plaque is observed microscopically

b) an emergency diagnosis on samples collected from animals with clinical signs of FMD: vesicular fluids from such an acutely infected animal contain high virus loads, which give high OD values that reduce the uncertainty of the test.

In the current method, the probability calculation is based on the normal distribution of the residuals of the analysis of variance (ANOVA). For this reason, the normality assumption has to be checked level by level, and any deviation could theoretically impair the validity of the model. In practice, however, the scientist must particularly focus on the values around the assay cut-off; slight deviations for high positive or low negative test results are more acceptable. The imprecision related to lack of normality can indeed be neglected when looking at samples with a probability/certainty of more than 99.9%, such as the high concentrations of antibodies in the first two serum dilutions analysed by the SPCE. Nevertheless, we recommend that special attention be paid to models that are associated with lack of normality, especially in situations where 'large' deviation from the cut-off is required to obtain acceptable levels of uncertainty. Furthermore, interpolation of probabilities for any possible result falling within the detection range of the test depends on the fit of the logistic model. Biased estimates of uncertainty may be obtained if the fit of the logistic model is poor. If the logistic model does not fit the data, it is advisable to use multiple link functions, such as the logit, the probit or the complementary log-log, and select the link with the best fit.

Uncertainty of measurement, as defined in the present paper, has the advantage of having as output a quantitative figure that is easily understood by the client. The method provides the scientist with a probability value for observing

the same qualitative result again if the test were to be conducted a second time. Such information is useful in deciding whether to retest the sample, to confirm the result using another test or to ask a different laboratory for confirmation. Moreover, the probability of observing the same qualitative test result when performing the same test twice under standardised repeatability conditions (same laboratory) could be interpreted as obtaining an 'analytical' predictive value of the test. However, the authors do not recommend using this term (or the expression 'probability of a positive test result') because it might be confusing to the client. Indeed, the laboratory must ensure that the client cannot confuse the uncertainty calculated using the current method with the true (or diagnostic) predictive value, which is calculated based on the sensitivity and specificity of the test in combination with the disease prevalence.

The suitability of the method described here was demonstrated for a competitive and an indirect ELISA, which differed significantly from each other. The method has also been carried out on several other ELISA tests performed at the Veterinary and Agrochemical Research Centre (VAR), for example the HerdChek® PRVgB Antibody Test Kit (Idexx), the POURQUIER® ELISA Leukosis Serum and the POURQUIER® ELISA Leukosis Milk (Institut Pourquier), and has been shown to be suitable for all the assays investigated. The results presented here suggest that the method is suitable for tests with either a fixed or a variable cut-off. It is therefore expected that the current method will perform equally well on any kind of ELISA and on molecular tests with a continuous data output that can be transformed to a positive/negative result, such as real-time polymerase chain reaction (PCR).

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Incertitude des mesures dans les épreuves ELISA par compétition et ELISA indirecte

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Résumé

Les auteurs décrivent une méthode permettant d'estimer l'incertitude des mesures pour les distributions gaussiennes des résultats des épreuves immuno-enzymatiques (ELISA), en utilisant les épreuves ELISA par compétition et ELISA indirecte pour la détection des anticorps dirigés contre la fièvre aphteuse. La répétabilité des tests a été déterminée en analysant la variance avec effets aléatoires ; la normalité des valeurs résiduelles a également été vérifiée. Les déviations standard des valeurs prédictives individuelles obtenues ont été transformées en intervalles de confiance encadrant chaque valeur observée, puis en probabilité de se trouver en-deçà ou au-delà d'un seuil limite. Dans un deuxième temps, des modèles de régression logistique ont permis d'interpoler des valeurs de probabilité pour la série entière des valeurs possibles du test. L'incertitude des mesures d'un résultat de test a été finalement définie comme la probabilité de ne pas obtenir le même résultat de test qualitatif en testant une nouvelle fois le même échantillon. S'agissant de l'ELISA par compétition, tout échantillon avec un pourcentage d'inhibition dépassant de 4 % le seuil limite est considéré affecté d'un niveau d'incertitude (c'est-à-dire d'une probabilité de donner un résultat négatif en répétant le test) inférieur à 5 %. S'agissant de l'ELISA indirecte, avec un seuil de densité optique (DO) de 0,1, l'incertitude est considérée inférieure à 5 % pour tout échantillon dont la DO normalisée est d'une valeur inférieure à 0,22.

Mots-clés

ELISA indirecte – ELISA de compétition (ECPS) – Fièvre aphteuse – Incertitude des mesures – Modèle de régression logistique – Répétabilité.

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Incertidumbre de las medidas con ELISA de competición e indirecto

J.F. Toussaint, P. Assam, B. Caij, F. Dekeyser, K. Knapen, H. Imberechts, N. Goris, G. Molenberghs, K. Mintiens & K. De Clercq

Resumen

Los autores describen un método para estimar la incertidumbre ligada a la medida de los resultados (con una distribución gausiana) del ensayo inmunoenzimático (ELISA), para lo cual se emplearon sendas pruebas ELISA, una de competición y otra indirecta, para la detección de la fiebre aftosa. Se determinó la repetibilidad del ensayo mediante un análisis de varianza de efectos aleatorios, y se comprobó que los valores residuales presentaran una distribución normal. El error típico (o estándar) de cada valor predicho fue transformado en intervalo de confianza alrededor del correspondiente valor observado, y convertido después en la probabilidad de situarse por encima o por debajo de un valor umbral. A continuación se utilizaron modelos de regresión logística para interpolar valores de probabilidad para todo el intervalo de posibles resultados del ensayo. Por ultimo, se definió la incertidumbre de la medida de una prueba como la probabilidad de que no se observe el mismo resultado cualitativo al repetir la prueba con la misma muestra. En el caso del ELISA de competición, toda muestra con un porcentaje de inhibición superior en un 4% al valor umbral presentaba un nivel de incertidumbre (probabilidad de resultado negativo en caso de repetición de la prueba) inferior al 5%. En el caso del ELISA indirecto, con un valor umbral de densidad óptica (DO) de 0,1, presentaban un nivel de incertidumbre inferior al 5% todas las muestras con un valor de DO normalizada superior a 0,22.

Palabras clave

ELISA de competición (ECFS) – ELISA indirecto – Fiebre aftosa – Incertidumbre de las medidas – Modelo de regresión logística – Repetibilidad.

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