Validation of an inhibition enzyme-linked immunosorbent assay system for the diagnosis of *Toxoplasma gondii* infection in buffaloes *(Bubalus bubalis)*

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Y. Armas Valdés [(1)], D. Obregón Álvarez [(1)], R. Grandía Guzmán [(2)], A. Mitat Valdés [(1)], E. Roque López [(1)], M. Pérez Ruano [(1)*] & A.A. Entrena García [(2)]

(1) Agricultural University of Havana (UNAH), Autopista Nacional Km 23½, Apartado 18, San José de las Lajas, Mayabeque, Cuba

(2) National Centre for the Production of Laboratory Animals, Finca Tirabeque, Bejucal, Mayabeque, Cuba

*Corresponding author: yarmas@unah.edu.cu

Summary

Toxoplasmosis is one of the most widespread zoonoses in the world owing to the existence of a wide variety of *Toxoplasma gondii* hosts, which include several domestic animal species. In Cuba, there is sustained production of the *Bubalus* species, which is highly adaptable and disease resistant, although it has been identified as a reservoir for a range of aetiological agents. Several countries have reported buffaloes as the intermediate host of *T. gondii* and have expressed the need to carry out epidemiological studies and confirm the possible presence of this parasitic infection in the *Bubalus* species. The current study was conducted to validate an inhibition enzyme-linked immunosorbent assay (i/ELISA) system for the diagnosis of *T. gondii* infection in buffaloes *(Bubalus bubalis)*. This involved evaluating its performance in relation to that of a latex agglutination test. With buffalo sera, the i/ELISA assay showed a sensitivity of 100%, a specificity of 99.5%,

and a concordance of 0.99 (considered very good) with respect to the reference diagnostic method. The conclusion is that i/ELISAperforms extremely well as a serological test for the diagnosis of *T. gondii* in buffaloes.

Keywords

Buffalo – i/ELISA – Inhibition enzyme-linked immunosorbent assay system – Latex agglutination – *Toxoplasma gondii*.

Introduction

Toxoplasmosis is a zoonotic infection of animals that is highly important because of its impact on both human and animal health. Epidemiological investigations have been carried out in veterinary science, especially over the past 60 years, with the aim of developing diagnostic systems, chiefly intradermal reaction tests and serological techniques such as complement fixation and enzyme-linked immunosorbent assay (ELISA) (1).

Before introducing any technique into the diagnostic network, quality assurance and quality control systems need to be established to ensure that the system is functioning properly and to confirm the quality of the data (2).

Assay validation is a fundamental step in ensuring that test results reflect the true status of the samples, it is in evaluating a diagnostic test that its suitability for a specific purpose is determined. To predict the efficiency of a diagnostic test, a validation methodology must be followed in order to document the expected analytical results from the test in question (2).

Some authors (3) describe the validation of diagnostic assays for infectious diseases as a set of interrelated and incremental processes that are duly documented and controlled, the results of which remain within statistically defined limits. These processes consist of standardisation, optimisation and performance evaluation.

In Cuba, *T. gondii* is usually diagnosed by complement fixation but, in recent years, the inhibition ELISA (i/ELISA) system has been developed as an alternative method, which has advantages in terms of its sensitivity and specificity, as well as from the economic standpoint.

The current study sets out to validate an i/ELISA system for the diagnosis of *T. gondii* infection in buffaloes (*Bubalus bubalis*) in Cuba's western region.

Materials and methods

Principle and protocol of the inhibition enzyme-linked immunosorbent assay system

The i/ELISA system was designed on the basis of the principle of inhibition of a single antibody. The technique begins with incubation of the test serum, which may contain low-affinity or low-concentration antibodies, in order to prioritise binding to the solid phase (coated with the soluble antigen made from the RH strain of *T. gondii*); next, the anti-*Toxoplasma* antibody conjugated to peroxidase (a bioreagent produced by the commercial firm SIGMA, which functions as both an enzyme conjugate and a detection antibody) is added; finally, the antigen is revealed using 10 mg of the buffer complex–chromogenic substrate o-phenylenediamine (OPD).

Interpretation of the assay results was based on the presence or absence of colour, with intensity of colour indicating the extent to which antibodies in the sample inhibited binding of the anti-*Toxoplasma* conjugate to the antigen immobilised on the plate, as follows.

– Negative reaction: characterised by an intense colour, indicating that the anti-*Toxoplasma* conjugate has bound to the antigen owing to the absence of specific antibodies in the sample.

– Positive reaction: characterised by absence of colour, indicating that binding of the anti-*Toxoplasma* conjugate to the antigen has been inhibited by antibodies present in the sample.

The test was developed by the parasitology laboratory of Cuba's national centre for the production of laboratory animals (CENPALAB). The sequence of operations making up this test was established as described by the World Organisation for Animal Health (OIE) (2), applying the following steps, in which the working volume is 100 µl per well:

i) Coat the wells with 100 µl of antigen, *T. gondii* RH strain, at 6 µg/ml, in a coating buffer and incubate for 16 hours at 4°C.

ii) Wash three times with 200 µl phosphate-buffered saline (PBS) containing 0.5% Tween 20 at pH 7.2 ± 0.2 (PBST) per well.

iii) Add 200 µl of blocking solution (PBS with 1% bovine serum albumin, 0.01% thiomersal and 1% glucose, pH 7.2 ± 0.2) per well to preserve the sensitised solid phase, and incubate for 1 hour at laboratory temperature (24°C).

iv) Add 100 µl of control sera diluted at 1/40 in PBST with skimmed milk at 1%, and incubate for 30 minutes at 37°C.

v) Wash four times with 200 µl PBST per well.

vi) Add 100 µl of rabbit anti-*Toxoplasma* immunoglobulin G (IgG) peroxidase conjugate diluted at 1/1000 in 1% PBST per litre, and incubate for 30 minutes at 37°C.

vii) Wash four times with 200 µl PBST (per well).

viii) Add 100 µl of buffer complex–chromogenic substrate (OPD).

ix) Stop the reaction with 100 µl of stop solution (2.5 N sulphuric acid [analytical purity]) after 15 minutes.

x) Read the optical density of the wells using a spectrophotometer at 492 nm.

Interpretation

The inhibitory factor (IF) was described as the percentage at which antibodies present in the test serum were able to inhibit binding of the conjugate. The following formula was used to calculate the IF:

IF = (OD of the sample / OD of the C (–)) × 100

Where OD = optical density and C (–) = negative control

– Any sample with an IF equal to or less than 15% was considered negative.

– Any sample with an IF of more than 15% and less than 20% was considered suspect.

– Any sample with an IF equal to or greater than 20% was considered positive.

Reference system used to evaluate the diagnostic performance of the inhibition enzyme-linked immunosorbent assay system

A commercial latex agglutination kit (Mascia Brunelli SpA) was used as a reference system to validate the i/ELISA results. The test protocol was as follows:

i) Remove from the refrigerator the serum samples and controls (positive and negative) to be used in the i/ELISA system and bring them to room temperature.

ii) Place 40 µl of undiluted sera (samples and controls) in a 6-well black-bottom plate.

iii) Place in the well close to the serum 20 µl of the undiluted, previously mixed latex solution sensitised with the purified soluble antigen of *T. gondii*.

iv) Mix the two reactants thoroughly using a plastic applicator and shake the black-bottom plate for 3 minutes gently and continuously.

v) Classify the samples.

Interpretation

Any samples in which veil-like clumping appeared were considered positive, taking as a reference the positive control, in accordance with the manufacturer's recommendations. The appearance of marked agglutination in the samples analysed indicated *T. gondii*-specific antibody concentrations exceeding 7.5 UI/ml. Any samples where no agglutination appeared were considered negative, taking as a reference the negative control.

Establishment of the reference panel

The formula proposed by Gardner for ELISA validation was used to determine the minimum sample size for inclusion in the reference panel.

$$N = \frac{Z^2 P(1-P)}{e^2}$$

Where:
N: sample size
P: analytical sensitivity
e: acceptable margin of error
Z: confidence interval

The analytical sensitivity of the assay was considered to be 99% (3) and a confidence interval of 95% was used for the calculation, with a 5% probability of error.

The reference panel was established with 400 buffalo sera from different categories of buffalo farm located in Cuba's western region.

A cross-sectional cluster sample survey was conducted. Most of the farms studied came under the controlled breeding system and none was situated close to human settlements, although they were close to other types of livestock farm and, in all cases, there was at least one house in the vicinity.

Sample collection

The blood was drawn by puncturing the jugular vein with a hypodermic needle and using the vacuum blood collection tube system. The capacity of the collection flasks (GPlast) was 10 ml. The blood was centrifuged at 1,200 rpm for 5 minutes to separate the serum, which was placed in 0.5 ml vials. The samples were identified and stored at -20°C until the time of analysis.

Performance evaluation of the inhibition enzyme-linked immunosorbent assay system

For the performance evaluation, the parameters established in Cuba were taken into consideration (4).

Precision studies (repeatability and intermediate precision)

To test the repeatability and intermediate precision of i/ELISA, four assays were performed using a replicate of each control serum (negative and positive, of low and high titre, respectively) in each assay, and the coefficient of variation (CV) of the results was determined to make a comparison between them.

Precision and accuracy studies

For the precision and accuracy studies, an evaluation was carried out to determine the dispersion of the results, i.e. to determine how far the data obtained in 25 assays using the control sera diverged from the averages. This dispersion was represented by control charts (average chart and range chart) and a range of ± 2 standard deviations was established as a limit, taking as a reference the overall average of all the assays (1).

These parameters were determined using the negative and positive controls (low titre and high titre, respectively), which were generated by binding or mixing six sera to obtain a final volume of 10 ml. They were then analysed by indirect fluorescent antibody test (IFAT) and latex agglutination to determine the presence of antibody. The control sera were identified as follows:

– the negative control: C (-)

– the low-titre positive control: C (+) IFAT 1/32, latex 1/16–1/32

– the high-titre positive control: C (++) IFAT 1/128, latex 1/64–1/128

These sera were processed in 25 consecutive assays with 5 replicates per assay, generating control charts of averages and ranges (variability). The assays were performed by the same operator over several days.

Concordance study

To evaluate the performance of the test, its concordance with the latex agglutination test was analysed.

The (relative) diagnostic sensitivity, (relative) diagnostic specificity, positive and negative predictive values, efficiency and Kappa coefficient were determined, in accordance with the recommendations (2, 3, 4, 5). A contingency table was used to organise the results (see Table I).

The parameters evaluated were calculated by the method described by Ochoa (5), using the following formulas:

– Relative sensitivity = [a / (a + c)] × 100.

– Relative specificity = [d / (d + b)] × 100.

– Positive predictive value = [a / (a + b)] × 100.

– Negative predictive value = [d / (c + d)] × 100.

– Efficiency = [(a + d) / (a + b + c + d)] × 100.

The level of concordance between the i/ELISA results and the reference method results was determined by means of the Kappa coefficient (K), which is calculated using the following formula (5):

$$K = (po - pe) / (1 - pe)$$

Where:

$$po = (a + d) / n \qquad pe = (P + N) / n \qquad n = a + b + c + d$$

Concordance between positive results:

$P = [((a + b) / n) \times ((a + c) / n)] \times n$

Concordance between negative results:

$N = (c + d) – ((a + c) – P)$

The results provided by the Kappa coefficient of concordance were classified in accordance with the reference literature (5), as shown in Table II.

Statistical processing of the results

The MINITAB® 18 statistical software (6) was used to process the data generated by the experiment, with a 95% confidence interval.

The Kolmogórov-Smirnov test (7) was used to determine if the distribution of results was normal, and Levene's test (7) was used to check them for homogeneity of variance. The precision studies determined the mean, standard deviation and intra- and inter-assay CV of the controls, and an acceptable limit of 10% CV was assumed, in line with the recommendations (3, 5).

In the precision and accuracy studies, the results of the 25 assays were processed to generate control charts of averages and ranges (variability). The criterion established for considering the system to be precise was that the values obtained from the different assays should lie within ± 2 standard deviations of the overall average of all the assay results (8).

Results and discussion

The four assays (Tables III and IV) resulted in stable values for the mean, standard deviation and CV of the control sera.

The CV (Table IV) showed less than 10% variability between replicates in the intra- and inter-assay studies, indicating that the conditions selected for i/ELISA guaranteed reliable results in terms of repeatability and intermediate precision, which agrees with the results obtained by other authors (3, 5, 9).

In the precision and accuracy studies, as the results of the statistical analysis confirmed that the data generated followed a normal distribution pattern, it was appropriate to prepare control charts to evaluate the dispersion of results by means of standard deviations, in accordance with established procedures (1, 8).

Figure 1 shows the results of the internal quality control using control charts, mean values and variability for each control serum. In each chart, limits of up to ± 3 standard deviations (standard limits, or SL) are indicated with red lines.

As an analysis of the control charts showed that variability of the results obtained in the replicates of the assays using the negative and positive controls remained within the limits set in the experiment (± 2 standard deviations) − results that agree with the models proposed for ELISA systems (3, 5) − the i/ELISA system is considered capable of generating precise and accurate results.

Several serological methods for the diagnosis of *T. gondii* have been recognised internationally, including the Sabin-Feldman dye test (reference system). However, the dye test has the disadvantage of using live parasites, meaning that it can be carried out only in specialised laboratories (10). For this reason, other methods have been recommended, such as latex agglutination, which is practical, sensitive, easy to perform, low-cost and capable of detecting both IgG antibodies and immunoglobulin M (IgM) antibodies, making it especially useful in epidemiological monitoring (11). All these advantages have led to latex agglutination being used worldwide to diagnose *T. gondii* in different animal species (12, 13).

In view of the above, a commercial latex agglutination kit was chosen as the reference method. According to an evaluation by Cuba's national reference laboratory for toxoplasmosis at the Pedro Kouri Institute of Tropical Medicine, this method gives reliable results (as confirmed by IFAT) and is the method used in the Cuban Ministry of Health network and in experimental species (1).

The contingency table with the results generated after processing the 400 samples analysed by i/ELISA and the latex agglutination test found only one discordant sample (one false positive), as Table V shows.

Table VI shows the results of an evaluation of the i/ELISA system's performance in analysing the 400 buffalo sera, using the latex agglutination system as a reference method.

The diagnostic sensitivity and specificity results achieved by i/ELISA validate the efficiency of this technique for the diagnosis of *T. gondii* in different animal species. These results agree with those found by Entrena (1), who evaluated the diagnostic performance of this technique in similar conditions with sera from dogs, rabbits, sheep, and various non-human primates, and determined that the sensitivity was 99.3% and the specificity was 99.6%.

These results also agree with those obtained by Morris and Kelly (14), using IFAT, a method with an equivalent sensitivity and specificity to those of the dye test (reference method), which are 99% and 100% respectively. The results were higher than those obtained by Lourenço *et al*. (15), who used immunoblotting for the diagnosis of congenital toxoplasmosis and found a sensitivity of 73.5% and a specificity of 97.4%. They were also higher than those of Dubey *et al*. (16), who used the microagglutination technique, obtaining a sensitivity of 58.3% and a specificity of 96.1%.

The predictive values change dramatically, while sensitivity and specificity remain unchanged. The predictive values obtained with i/ELISA, both positive and negative (99.5% and 100%, respectively), are higher than those recommended by Jacobson (3), who proposes that when prevalence decreases, the negative predictive value increases and the positive predictive value decreases.

Efficiency is the proportion of positive and negative cases that are correctly diagnosed (3, 5). I/ELISA diagnosed 99.8% of the positive and negative cases correctly, a result that supports the usefulness of this system for the diagnosis of *T. gondii* in buffalo. As there was only one

false negative, this result is close to the optimum efficiency of a test (where 100% of cases are correctly diagnosed) (5).

Analysis of the Kappa coefficient revealed a high level of concordance (0.99) with the reference diagnostic method (Table VI), and Ochoa considered it very good, being similar to the 0.98 found by Entrena (1).

In general, the results obtained agree with those of Dubey (10), who recommends the use of ELISA in the diagnosis of *T. gondii* in bovids, owing to its high diagnostic sensitivity and specificity. The results also agree with those obtained by Entrena (1), who evaluated the diagnostic performance of this system with serum panels from four different species (non-human primates, sheep, rabbits and dogs).

Of the 400 buffalo sera included in the validation study, 221 contained anti-*Toxoplasma* antibodies according to the i/ELISA used, which is a seroprevalence of 55.3%. These results were confirmed by latex agglutination test applied to the same samples during the validation of i/ELISA, with latex agglutination results agreeing with those of i/ELISA in 99.8% of cases.

This is the first finding regarding the prevalence of *T. gondii* in buffalo in Cuba, and it corresponds with the findings in other countries in which the presence of anti-*Toxoplasma* antibodies in buffaloes has been described, including Brazil, China, India and Italy.

Conclusions

This study validated the i/ELISA system for the diagnosis of *T. gondii* infection, making it an effective tool for the diagnosis of *T. gondii* antibodies in buffaloes.

I/ELISA demonstrated excellent diagnostic performance with the buffalo sera analysed.

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Table I
Contingency table used in the selection of the cut-off value and in the performance evaluation studies

i/ELISA system	Reference diagnostic method: latex agglutination		Total
	Positives	Negatives	
Positives	TP (a)	FP (b)	a + b
Negatives	FN (c)	TN (d)	c + d
Total	a + c	b + d	a + b + c + d

Where:

TP (a) = true positives or correctly detected positives

FP (b) = false positives

FN (c) = false negatives

TN (d) = true negatives or correctly detected negatives

i/ELISA: inhibition enzyme-linked immunosorbent assay

Table II
Level of concordance according to the Kappa coefficient

Concordance between the results obtained with two different tests	Kappa coefficient value
Slight	< 0.20
Fair	0.21 – 0.40
Moderate	0.41 – 0.60
Substantial	0.61 – 0.80
Almost perfect	0.81 – 1.00

Table III
Behaviour of the mean and standard deviations of results obtained when using controls in the inhibition enzyme-linked immunosorbent assay system

Controls	Assay 1		Assay 2		Assay 3		Assay 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C (-)	0.546	0.0099	0.489	0.0085	0.544	0.0035	0.575	0.0184
C (+)	0.196	0.0113	0.186	0.0092	0.169	0.0078	0.233	0.0113
C (++)	0.185	0.0106	0.190	0.0106	0.164	0.0035	0.218	0.0184

C (-) = negative control

C (+) = positive control

C (++) = high-affinity positive control

SD = standard deviation

Table IV
Results of precision studies of the inhibition enzyme-linked immunosorbent assay system

Controls	Coefficients of variation (%)				
	Repeatability				Intermediate precision
	Assay 1	Assay 2	Assay 3	Assay 4	
C (-)	1.8	1.7	0.7	3.2	5.6
C (+)	5.8	5.0	4.6	4.9	7.9
C (++)	5.7	5.6	2.2	8.4	7.9

C (-) = negative control

C (+) = positive control

C (++) = high-affinity positive control

Table V
2 × 2 contingency table using two methods: the inhibition enzyme-linked immunosorbent assay system and latex agglutination

i/ELISA system	Reference diagnostic method: latex agglutination		Total
	Positives	Negatives	
Positives	211	1	212
Negatives	0	188	188
Total	211	189	400

i/ELISA: inhibition enzyme-linked immunosorbent assay

Table VI
Results of the validation of the inhibition enzyme-linked immunosorbent assay system

Parameter	Value
Sensitivity (%)	100
Specificity (%)	99.5
Positive predictive value (%)	99.5
Negative predictive value (%)	100
Efficiency (%)	99.8
Kappa coefficient	0.99

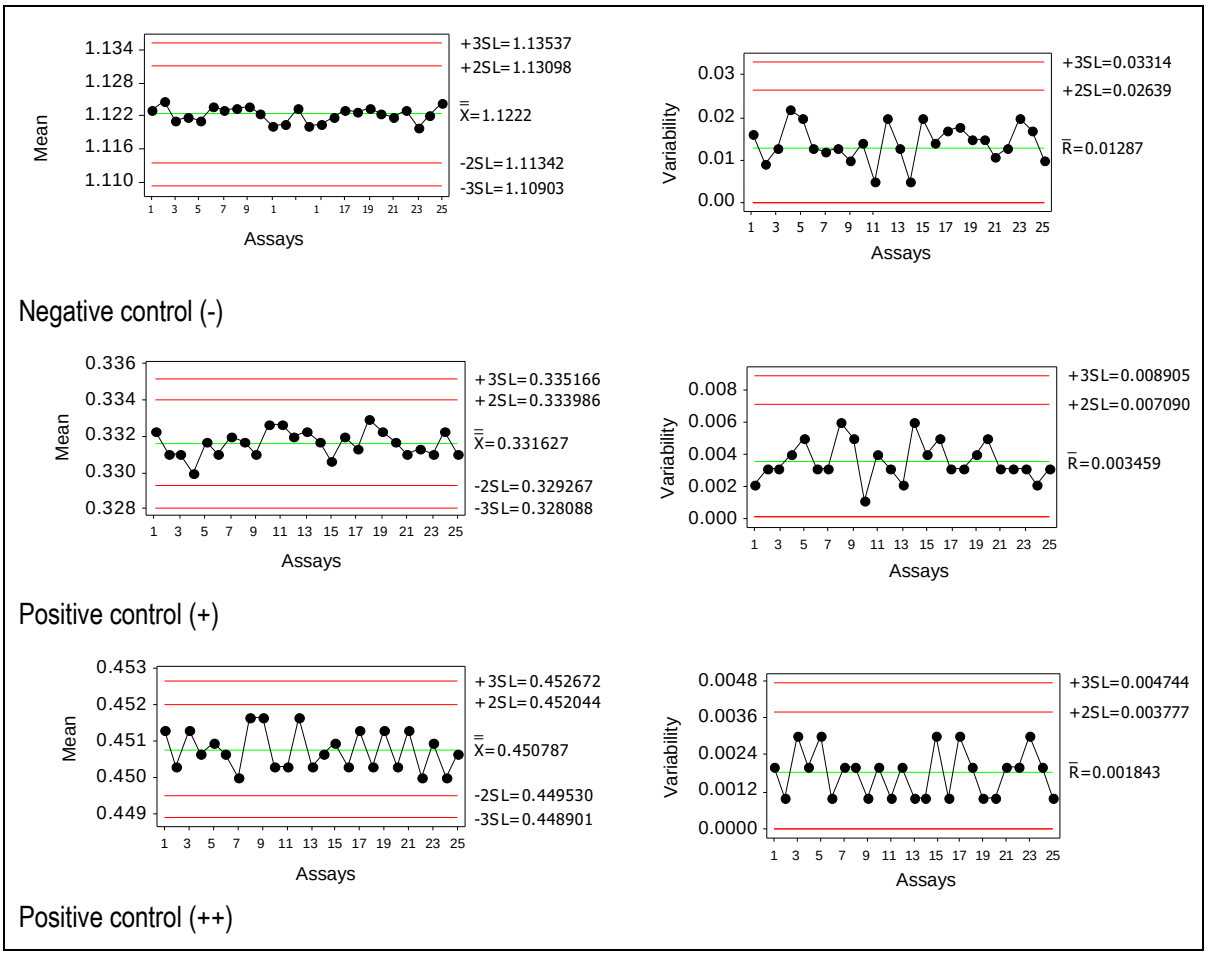

Fig. 1

Results of the precision and accuracy studies

X: mean

SL: standard limitations

R: rank