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Short communication

Assessment of the repeatability and border-plate effects of the B158/B60 enzyme-linked-immunosorbent assay for the detection of circulating antigens (Ag-ELISA) of *Taenia saginata*

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ABSTRACT

The monoclonal antibody-based circulating antigen detecting ELISA (B158/B60 Ag-ELISA) has been used elaborately in several studies for the diagnosis of human, bovine and porcine cysticercosis. Interpretation of test results requires a good knowledge of the test characteristics, including the repeatability and the effect of the borders of the ELISA plates. Repeatability was tested for 4 antigen-negative and 5 antigen-positive reference bovine serum samples by calculating the Percentage Coefficient of Variation (%CV) within and between plates, within and between runs, overall, for two batches of monoclonal antibodies and by 2 laboratory technicians. All CV values obtained were below 20% (except one: 24.45%), which indicates a good repeatability and a negligible technician error. The value of 24.45% for indicating the variability between batches of monoclonal antibodies for one positive sample is still acceptable for repeatability measures. Border effects were determined by calculating the %CV values between the inner and outer wells of one plate for 2 positive serum samples. Variability is a little more present in the outer wells but this effect is very small and no significant border effect was found.

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1. Introduction

Taenia is a genus of worldwide occurring cestodes, including species such as *Taenia saginata* and *Taenia solium*, of which the adult tapeworm resides in the intestinal lumen of the final host (taeniosis) and the metacestode larvae occur in the intermediate host as cysticerci (cysticercosis). A number of species within this genus can have a great impact on public health (e.g. *T. solium*) and may account for great economic losses due to condemnation of infected meat (e.g. *T. saginata*) (Dorny et al., 2009).

Current control measures tackling the intermediate host are primarily focussed on official meat inspection, which, in Europe, is performed according to EU regulation (EC directive 854/2004). For bovines, meat inspection has a known low sensitivity (<15%) (Kyvsgaard, 1990; Dorny et al., 2000), indicating a need for improved diagnostic techniques to detect bovine cysticercosis.

One of the serological tests used for this purpose (but still only for research) is a monoclonal antibody-based circulating antigen detecting ELISA (B158/B60 Ag-ELISA), developed by Brandt et al. (1992) and Van Kerckhoven et al. (1998) and modified by Dorny et al. (2000). The monoclonal antibodies were developed against the excretory-secretory antigens of the metacestode stage (cysticerci) of the bovine tapeworm *T. saginata* with the aim to detect only viable cysticerci in cattle, as these are of public health concern. The monoclonal antibodies used in the sandwich ELISA show genus, but not species specificity, so the ELISA can also detect viable cysticerci of *T. solium* in humans and pigs, *T. hydatigena* in pigs (Dorny et al., 2004a) and *T.ovis* in sheep (Brandt et al., 1992).

This test has been used elaborately in several studies for the diagnosis of human, bovine and porcine cysticercosis (Dorny et al., 2000; Dorny et al., 2004b; Allepuz et al., 2012; Eichenberger et al., 2013). Interpretation of the test results requires a good knowledge of the test characteristics. Repeatability is an estimate of precision in an assay. It is the degree of matching between repeats of measurements within and between 'runs' of the same test in a certain laboratory (OIE, 2013). It is essential to be able to compare results

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of a test in time to give a meaningful interpretation to experimental and observational data.

The objective of this study was to determine the repeatability of the B158/B60 Ag-ELISA, tested for reference bovine serum samples collected in Belgium. Furthermore, a trial was conducted to evaluate the uniformity of the test results within one plate. It is often stated that the outer wells can be more influenced by temperature than the inner wells (Venkatesan and Wakelin, 1993). To test for this effect of the border of the ELISA plates, the results from the outer wells are compared with those from the inner wells of the ELISA plate.

2. Materials and methods

2.1. Test design repeatability trial

Five positive (P) and four negative (N) bovine serum samples, part of a serum bank present in the laboratory of the department of Biomedical Sciences at the Institute of Tropical Medicine, Antwerp, Belgium, were used to test repeatability of the Ag-ELISA. Positive samples (3 high, one medium and one low positive) originated from experimentally infected animals (under ethical clearance number: EC2012/133) and negative samples were collected at Belgian slaughterhouses from calves raised for white veal production. Samples were randomly tested in several replicates (10/plate) on different ELISA plates (2 plates per run, 10 runs) to determine repeatability within and between plates and runs. The test was repeated for two combinations of batches of monoclonal antibodies (IgG1 antibodies $B158C_{11}A_{10}$ and $B60H_8A_4$, produced by Eurogentec), to determine repeatability between batches and by a different technician to test technician error (for the first combination of monoclonal antibody batches).

2.2. Test design border effects trial

Two reference positive bovine serum samples (S1, S2) were tested on two ELISA-plates to test for uniformity within one plate. An identical serum sample was added to all wells in one plate, with the exception of two times the substrate control (SC) and conjugate control (CC).

2.3. Enzyme-linked-immunosorbent assay for the detection of circulating antigens (Ag-ELISA)

The Ag-ELISA was performed as described by Dorny et al. (2000). Briefly, pre-treatment of the serum samples was done by mixing an equal volume of serum and freshly prepared 5% trichloroacetic acid (TCA) (Sigma-Aldrich) w/v in distilled water and incubation of this mixture for 20 min at room temperature, followed by centrifugation for 5 min at 10,000*g*. An equal volume of a sodium carbonate/bicarbonate buffer (0.610 M) at pH 10.0 was added to the supernatant to raise the pH.

The actual test started with coating the polystyrene ELISA plates (Nunc® Maxisorp) with 100 μl of MoAb $B158C_{11}A_{10}$ (at a protein concentration of 5 μg ml^{-1}) in carbonate buffer, pH 9.6 per well. Incubation was 30 min at 37 °C while shaking, followed by washing one time with 0.05% Tween 20 (Sigma-Aldrich) in phosphate buffered saline (pH 7.2) (PBS-T20). Blocking was done with 150 μl per well of 1% newborn calf serum (NBCS, Fisher Scientific) in PBS-T20 (incubation 15 min at 37 °C while shaking). Plates were emptied, but not washed, before adding 100 μl of the pre-treated test sera (1/4 dilution of original sample) (incubation 15 min at 37 °C while shaking). After washing 5 times, 100 μl of the second biotinylated MoAb $B60H_8A_4$ (1.25 μg ml^{-1} in 1% NBCS in PBS-T20) was added to each well (incubation 15 min at 37 °C while shaking, washing 5 times). Next, 100 μl of streptavidin-horseradish peroxidase (Jackson-Immunoresearch) diluted 1/10000 in 1% NBCS in PBS-T20 was added (incubation 15 min at 37 °C while shaking, washing 5 times). Finally, 100 μl substrate solution consisting of 1.2-phenylenediamine dihydrochloride (OPD) in citrate buffer (pH 5.2) (Dako) and H_2O_2 was added and after incubation (15 min in dark), the reaction was stopped with 50 μl 4 N H_2SO_4. The plate was read using an automated spectrophotometer (Titertek Multiskan EIA reader) at 492 nm with a reference at 655 nm.

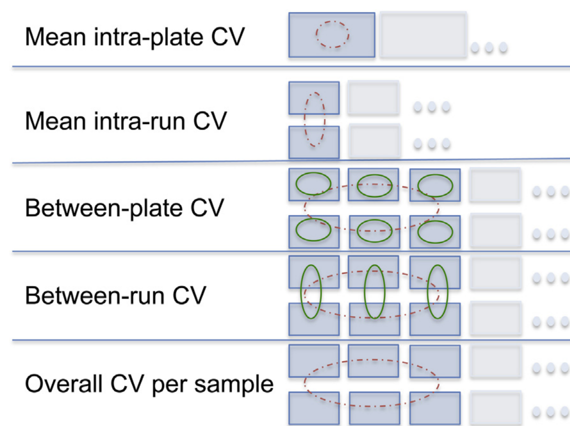

Fig. 1. Illustration of the different coefficients of variation (CV) determined.

The cut-off value is calculated based on the OD values of the negative samples using a variation of the students *t*-test at a probability level of P = 0.001 (Sokal and Rohlf, 1991). The optical density of each serum sample was compared with this cut-off value to determine the result of the test.

2.4. Analyses

The results were calculated using Microsoft Excel. Repeatability is expressed through a Percentage Coefficient of Variation (%CV), namely the standard deviation (SD) expressed as a percentage, over the mean ($\bar{x}$) of the replicates, in agreement with ISO practices (International Organization for Standardization) (ISO 5725: 1994).

$$\%CV = \frac{SD}{\bar{x}} \times 100$$

Adequate repeatability is indicated by %CV values <20%. If evidence exists for excessive variation (>30%), more preliminary studies are required to determine whether stabilization of the assay is possible, or whether the format should be abandoned (Jacobson, 1998; Pryseley et al., 2010). Variation coefficients were determined within and between plates, within and between runs, overall (Fig. 1), between batches and between technicians (technician error).

While analysing ELISA results using the optical density (OD) values, it is important to keep in mind that, although a difference in OD value between two measurements of the same sample may not be within an acceptable range from a statistical point-of-view, this difference may be negligible from an ELISA technical point-of-view.

Variability between inner and outer wells (border effects) were tested by comparing OD readings of the inner wells (separated at least two wells from the border), with the OD readings from the outer wells by comparing the %CV values between inner and outer wells. This was done in the same manner as the calculation of the mean intra-run %CV, but with the inner and outer wells considered as the separate 'runs'.

Even tough %CV values were calculated for both negative and positive samples, repeatability measurements are not meaningful

Table 1
Results (percentage coefficients of variation (%CV)) for the repeatability of the Ag-ELISA for batch 1 and batch 2 for technician 1, p1-5: reference positive samples.

	Sample	Mean intra-plate %CV	Mean intra-run %CV	Between plate %CV	Between run %CV	Overall %CV
Batch 1	p1	3.66	3.94	7.70	7.73	8.32
	p2	5.29	5.88	10.55	10.35	11.53
	p3	6.65	7.46	15.31	15.16	16.43
	p4	8.96	9.41	18.11	18.34	19.93
	p5	8.07	8.52	16.13	16.24	17.70
Batch 2	p1	4.39	5.32	7.00	6.38	8.08
	p2	7.09	9.22	12.57	10.91	14.11
	p3	9.11	10.05	9.94	8.83	13.17
	p4	12.02	13.74	11.15	9.06	16.86
	p5	9.12	11.74	13.14	10.59	15.92

Table 2
Results (percentage coefficients of variation (%CV)) for the repeatability of the Ag-ELISA for batch 1 (technician 2).

Sample	Mean intra-plate %CV	Mean intra-run %CV	Between plate %CV	Between run %CV	Overall %CV
p1	2.78	3.50	5.63	5.30	6.15
p2	4.82	5.38	6.55	6.27	8.00
p3	6.03	6.82	8.20	7.81	10.04
p4	7.97	9.38	9.52	8.30	12.21
p5	7.55	8.61	8.16	7.10	10.86

Table 3
Results (percentage coefficients of variation (%CV)) for the repeatability of the Ag-ELISA between batches for technician 1 and between technicians for batch 1.

Sample	Between batch %CV	Between technician %CV
p1	3.40	4.72
p2	11.24	3.20
p3	14.12	6.04
p4	14.05	6.32
p5	24.45	2.10

for values approaching zero (negative samples), so only %CV values for the positive serum samples are presented (Jacobson, 1998; Pryseley et al., 2010; OIE, 2014).

3. Results

3.1. Repeatability

ELISAs were performed for the first and second combination of the monoclonal antibodies by one technician (Table 1). The coefficients of variation remained below 20% for all positive samples for all five %CV values calculated (mean intra-plate, mean intra-run, between plate, between run and overall), indicating a good repeatability.

The ELISA was repeated by a second laboratory technician for the first combination of antibody batches (Table 2). Once again, all %CV values were smaller than 20% for the positive test samples.

The results of the repeatability analysis to determine the difference between two batches of monoclonal antibodies are depicted in the first column of Table 3. For the positive samples p1 to p4, %CV is smaller than 20%, but p5 (moderately positive sample) had a coefficient of variation of 24.45%.

Table 3 shows the %CV values of repeatability between the two technicians for the first combination of antibody batches in the second column. The technician error appears to be very small, since all %CV values are below 10%.

3.2. Border effects trial

For both samples, all %CV values (intra-plate, intra-inner wells, intra-outer wells and between inner and outer wells) are very low (Table 4).

Table 4
Results (percentage coefficients of variation (%CV)) for the border effects for sample 1 (S1) and 2 (S2).

	%CV S1	%CV S2
intra-plate	5.47	5.92
intra-inner wells	3.83	3.18
intra-outer wells	6.70	4.49
between inner-outer wells	5.27	3.84

4. Discussion

The ELISA had a high repeatability for all tested possibilities: intra-plate (2.78–12.02%CV), intra-run (3.5–13.74%CV), between-plate (5.63–18.11%CV), between-run (5.3–18.34%CV) and overall (6.15–19.93%CV) (Tables 1 and 2). All values of %CV for positive samples were within the standard empirical criteria of less than 20% for raw values, suggested by Jacobson (1998).

As mentioned before, %CV values will always be bigger for values approaching zero (negative samples). The amount of variation will appear much bigger in comparison with a very small value, than compared with a bigger value, making it impossible to get valuable information from the %CV values of negative samples.

ELISA results depend heavily on the characteristics of the antibodies used in the test system. The results of the between batches repeatability showed that the test results obtained by using different combinations of antibody batches, performed at different times, had highly repeatable results. Between-batch repeatability varied between 3.4 and 14.12%CV for samples p1 to p4 (Table 3). Even though the CV value for sample p5 between batches was 24.45%, this is not considered as excessive variation (>30%) and repeatability can still be accepted (Jacobson, 1998). Technician error, analysed as between technician %CV, was very small for all positive samples (2.10–9.8%) (Table 3), indicating no significant technician effect on the results.

For the border effects trial, %CV was first calculated for the complete plates (intra-plate). For S1, this value was 5.47% and for S2 5.92%, confirming the intra-plate repeatability found in the repeatability trial.

When comparing the intra-inner wells %CV (S1: 3.83% and S2: 3.18%) with the intra-outer wells %CV (S1: 6.7% and S2: 4.49%), it is found that the %CV for the outer wells is a little higher indicating that there is more variability in the OD values of the outer wells. This effect is however very small and the %CV of the outer wells and

between the inner and outer wells for both samples (S1: 5.27%, S2: 3.84%) are very low, indicating there is no important effect between inner and outer wells.

In conclusion, test results indicate that the B158/B60 Ag-ELISA performed on bovine serum samples has a good repeatability on all levels. Different technicians can perform the test without an unfavourable effect on the results. The border effect in the variability of OD values remains within an acceptable interval. The B158/B60 Ag-ELISA needs to be further evaluated and characterised (sensitivity, specificity). Even though the same test with the same monoclonal antibodies is performed on human and porcine samples, care has to be taken with extrapolating these results of repeatability to the use of this Ag-ELISA on samples from other species.

Conflict of interest statement

None of the authors has a financial or personal relationship with other people or organisations that could inappropriately influence or bias this paper.

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