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Original Research Paper

METHOD AGREEMENT OF QUANTITATIVE MEASUREMENTS – STABILITY OF BUTANOL EXTRACTS OF RESAZURIN AS A MODEL

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Summary: A resazurin reduction assay depends on the ability of metabolically active spermatozoa to reduce resazurin-redox dye to resorufin, which may then provide valuable information for predicting sperm fertilizing capacity. We investigated whether it was possible to accurately measure the resazurin reduction in butanol extracts a day and as late as a week after an assay was performed on boar semen.

According to the scatter diagrams with fitted regression line, it was evident that the paired measurements, i.e. A_{610} at day 0 and day 1 as well as A_{610} at day 0 and day 7, were close to the line of equality. It was also established that the percentage biases were in the range of the within-run coefficient of variation. From the absolute bias plots, it was evident that there was no proportional bias. In order to assess how well the measurements agreed at the individual level, we also determined the limits of agreement. More than 95% of the absolute differences were within the limits of agreement, confirming that the level of agreement between the methods was satisfactory for both of the investigated comparisons.

The stability of the butanol extracts confirmed that the resazurin reduction could be spectrophotometrically measured up to one week after an assay was performed. The usefulness of the assay is, therefore, greatly enhanced as it can be used in on-farm AI laboratories that do not have immediate access to spectrophotometers.

Key words: semen - analysis; comparative study; statistics - methods; fertility agents - analysis

Introduction

Traditionally, boars have been chosen as AI (artificial insemination) studs based upon their genetic excellence. More often than not, these genetic merits are related to improved meat quality and days to market rather than to reproductive performance. A routine examination of a boar's semen quality is very important as an insurance against reproductive disorders. The costs arising from using poor quality semen are high, as it has a negative impact on a herd's farrowing rate, its litter size and non-productive days as well as on the culling of sows and gilts (1).

Although various analytical techniques have been developed to evaluate sperm quality, including sperm concentration, motility, viability and morphology, there is no single method that provides a complete picture about semen quality (2, 3). Another important issue is that for some on-farm AI laboratories, these same routine semen evaluations tend to be impractical because of limitations of equipment, skilled laboratory staff or time. Therefore, a reliable, simple, cost effective and rapid method of assessing the quality of boar spermatozoa would be of benefit to both livestock producers and veterinary practitioners (4).

Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) is a redox dye used as an indicator of dehydrogenase activity (4, 5). Vital spermatozoa produce reducing factors such as NADH + H^+ during the metabolic process of glycolysis and the citric-acid cycle, and these factors participate in the redox reaction. The diaphorase enzyme transfers electrons to the resazurin dye, which becomes reduced to resorufin and then to dihydroresorufin and manifests as a visual colour change from blue to pink and then to white (6, 7). We developed a spectrophotometric application of resazurin reduction assay for

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boar semen to quantitatively measure the change from blue to pink in butanol extracts (8). After developing the assay, we wondered if it was possible to measure the absorbance at a later date, i.e. within a day or even a week of the assay. In this study, we evaluated the simila-rity between the measurements of absorbance of butanol extracts measured immediately following an assay (day 0), 24 hours after the assay (day 1) and a week after the assay (day 7). Moreover, a comparison between two clinical laboratory met-hods was required because we adapted an existing method to make it more convenient to use. A sa-tisfactory level of agreement would indicate that the modification was successful, which in turn would greatly enhance the usefulness of the assay as it could then be performed even if a spectrophotometer was not immediately available.

Material and methods

Resazurin reduction assay

The resazurin reduction assay was performed as previously described (8). Briefly, 30-µl of 1.8 mM resazurin (Sigma, Germany) diluted in physiological saline was added to 3 ml semen sample (that had been maintained at 37 °C) diluted 1:2 with BTS semen extender (Beltsville Thawing Solution, Netherlands) and incubated at 37 °C in a water bath for 10 minutes. After incubation, two sub-samples of 1 ml were added to 1.5 ml of butanol (Merck, Germany).

Butanol extracts

After the resazurin reduction assays, the developed colour was extracted with butanol. After rapid vortexing, the samples were centrifuged at 3000 g for 10 minutes. The extract's absorbance at 610 nm was then measured in the clear, upper layer of butanol. There were 112 butanol extracts included in this study. We measured the A_{610} immediately (day 0), one day (day 1) and one week (day 7) after the assays were performed. In the meantime, the butanol extracts were kept at 4 °C.

Bias determination

While comparing the measurements between days 0 and 1 as well as between days 0 and 7, we developed scatter graphs to which we fitted regression lines in order to establish the type/s of bias that might be present. Absolute bias is present when data points lie a similar distance to one side of a 45° line in a conventional plot. Proportional bias is present when the distance of the data points from the 45° line increases with the measured value but would be negligible at zero, whereas combined bias is present when the data points diverge increasingly from the 45° line but indicate a measurable value on one axis when the other is zero (9). The least squares linear regression method is appropriate for statistically assessing absolute and/or proportional bias because the x values can be regarded as having no error. Linear regression is applied to paired measurements with the object of fitting the best straight line that can pass through the plotted points to predict the value of the dependent variable, to be expected at any value of the other independent variable (9).

Method agreement

The differences between the pairs of measurements – day 0/day 1 and day 0/day 7 – were calculated for absorbance at 610 nm for each butanol extract. In absolute bias plots, the biases were plotted against their average value for each sample. In order to assess how well the paired measurements agreed with each other, we determined the limits of agreement. The upper limit of agreement was calculated as being $\overline{d} + 2s_{diff}$ and the lower limit of agreement as $\overline{d} - 2s_{diff}$, where $\overline{d}$ was the mean of differences for all the samples (average bias) and s_{diff} was the standard deviation of the differences; $2s_{diff}$ is also referred to as British Standard Institution repeatability (or, reproducibility, as relevant) coefficient and indicates the maximum difference likely to occur between two measurements. This coefficient is the value below which the bias between paired results may be expected to lie with 95% certainty (10).

Results

Using linear regression, we described the relationship between absorbance on day 0 and absorbance on days 1 and 7, respectively, by determining the straight line that most closely approximates the data points on a scatter diagram. The scatter diagram of absorbance at 610 nm on day 0 and absorbance at 610 nm on day 7 is similar to that shown in Figure 1 (data not shown); the data, with a regression line fitted, corresponded to the regression equation: A_{610} (day

7) = 0.00164 + 0.99689 x A_{610} (day 0); R=0.99877, P<0.0001. The estimated linear regression equations indicate that the points are close to the line of equality, i.e. the 45° line.

The mean percentage bias was 0.996 ± 2.820% and 1.380 ± 8.056% for measurements of A_{610} on day 1 and day 7. In the relative bias plot (Fig. 3), a greater number of percentage biases were detected at low absorbance values. However, these biases are in the range of the within-run coefficient of variation of the test, calculated as 7.79 ± 4.06% (8). From the absolute bias plot (Fig. 2), it is also evident that the scatter of the points is random indicating that the size of the discrepancy between the two absorbance values is not related to the size of the absorbance. Therefore no proportional bias is detected.

Average absolute biases were close to zero and were calculated as 0.0045 ± 0.0066 and 0.0007 ± 0.0076 for the measurements of A_{610} on days 1 and 7, respectively. Measurements obtained on day 0 and those obtained on day 1 and day 7 agree; 99.1% and 95.54% of the differences lie within the limits of agreement, respectively.

Therefore, we can measure A_{610} of butanol extracts at any time up to one week after an assay is conducted, confirming the highly practical value of this method.

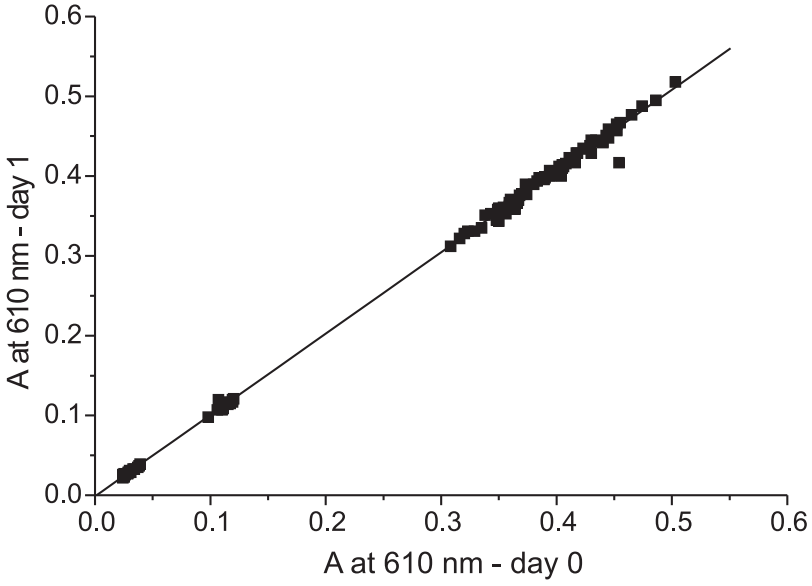

Figure 1: Scatter diagram of A_{610} on day 0 versus A_{610} on day 1 with regression line fitted
Regression equation: A_{610} (day 1) = -0.00125 + 1.01896 x A_{610} (day 0), R=0.99926, P<0.0001

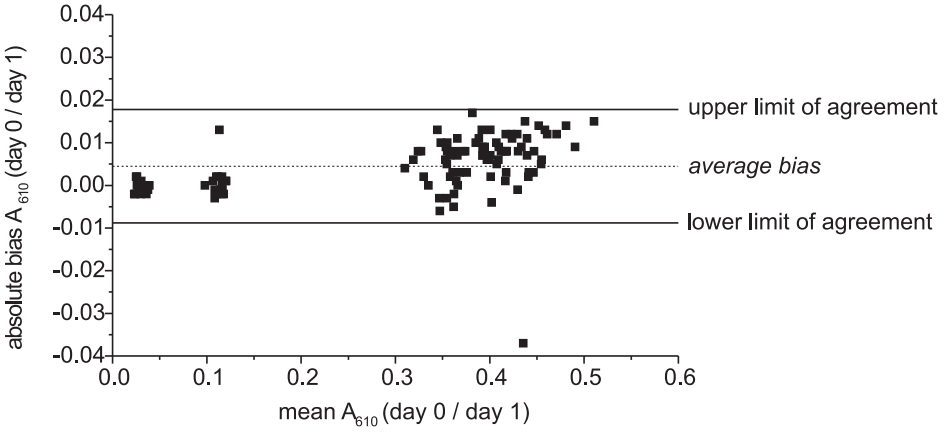

Figure 2: Absolute bias plot of A_{610} on day 0 versus A_{610} on day 1 showing average bias and limits of agreement

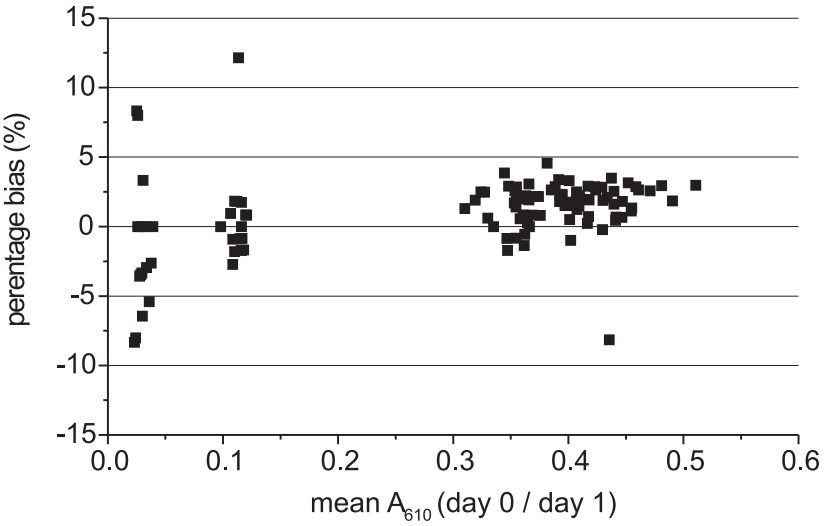

Figure 3: Relative bias plot of A_{610} on day 0 versus A_{610} on day 1

Discussion

It has been reported that the colour change in a resazurin reduction assay correlates significantly with the concentration of motile spermatozoa (5, 8, 11). A high degree of discrimination between good quality and poor quality boar semen samples was also achieved utilizing the sperm index (SI = sperm concentration multiplied by the square root of the percentage of motility multiplied by the percentage of normal morphology) (8). The colour change can be matched by a colour chart but varies between evaluators; therefore a spectrophotometric application of the resazurin reduction test is preferable, enabling the metabolic activity of the sperm to be measured quantitatively (12). It has been mostly used for the evaluation of human semen (6, 7, 12, 13, 14), however, to the best of our knowledge, it has only been used in veterinary medicine for evaluating the quality of ram (12) and boar (8) semen. Following Zalata et al. (7), we used butanol to extract the developed colour after the boar semen assay and measured the level of absorbance in the clear, upper layer of butanol, thereby eliminating the problem of sample turbidity. The resazurin reduction assay is reliable, easy to perform and does not require sophisticated laboratory equipment. On the other hand, a spectrophotometer is not standard laboratory equipment in the majority of on-farm AI laboratories. Therefore, we explored the possibility of measuring the absorbance up to a week after the assay had been performed.

It is essential to establish that a method is repeatable before comparing two measurements for reproducibility (10). The within-run coefficient of variation, calculated as 7.79 ± 4.06%, confirmed that the method had a satisfactory level of repeatability (8), therefore, the pairs of measurements of A_{610} were valid for comparison.

Scatter plots and absolute and relative bias plots give the best overview of comparative data (15, 16). Using scatter diagrams with regression lines fitted, we established that the paired measurements, i.e. A_{610} at day 0 and day 1 (Fig. 1) as well as A_{610} at day 0 and day 7, were close to the line of equality.

According to the available literature, a very common way of investigating method agreement is by performing a paired t-test or by calculating a correlation coefficient to provide a measure of the agreement, however, in this instance, neither method is appropriate for the reasons listed below (10). The paired t-test tests the null hypothesis that means the difference is zero. If the differences between the pairs are large – indicating that the methods do not agree – but are evenly scattered around zero, then we will obtain a non-significant result. We can only conclude that there is no bias, not that the methods agree. Correlation is a statistical method used for quantifying any association between two continuous variables (17). The correlation coefficient provides a measure of the linear association between the measurements obtained by the two methods. It gives us an indication of how close the observations in the scatter diagram are to a straight line. However, to assess agreement, we need to know how close the points are to the line of equality, i.e. the 45° line (10).

We were interested in assessing the similarity between A_{610} on day 0 and day 1 as well as

between day 0 and day 7, so we compared pairs of measurements. Therefore, we calculated the differences between A_{610} at day 0 and day 1 and between day 0 and day 7 for each butanol extract. The mean of these differences ($\overline{d}$) is an estimate of the average bias of one method relative to the other. If this bias is zero, then the two measurements agree on average. However, this does not imply that they agree for each individual measurement. In order to assess how well the measurements agree on an individual basis, we determined the limits of agreement (10). More than 95% of the absolute differences were less than the reproducibility coefficient, confirming that the level of agreement between the methods was satisfactory for both of the investigated comparisons. Therefore, we can measure the level of absorbance up to one week from the time that a test is performed. The usefulness of the assay is, therefore, greatly enhanced as it can be used in on-farm AI laboratories that do not have immediate access to spectrophotometers.

The resazurin reduction assay has been shown to be reliable, simple and easy to perform and would, therefore, be of benefit to both livestock producers and veterinary practitioners in evaluating the quality of boar semen.

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UJEMANJE METOD PRI KVANTITATIVNIH MERITVAH - STABILNOST BUTANOLNIH EKSTRAKTOV RESAZURINA KOT MODEL

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Povzetek: Redukcijski test z resazurinom temelji na sposobnosti metabolično aktivnih semenčic, da reducirajo redoksno barvilo resazurin v resorufin. Test lahko prispeva k ugotavljanju oploditvene sposobnosti semenčic. Ugotavljali smo možnost določanja redukcije resazurina v butanolnih ekstraktih dan ali celo teden po izvedbi testa.
Na podlagi razsevnih grafov s premico linearne regresije lahko ugotovimo, da so parne meritve, to je A_{610}, izmerjene na dan 0 in dan 1, kot tudi parne meritve, opravljene na dan 0 in dan 7, blizu premici enakosti. Relativne razlike med parnimi meritvami so znotraj koeficienta variacije za ponovljivost v testu. Grafi absolutnih razlik dokazujejo, da med primerjanimi metodami ni proporcionalnih napak. Ujemanje posameznih parnih meritev je zadovoljivo, kar potrjuje dejstvo, da je več kot 95 % absolutnih razlik znotraj meja ujemanja.
Merjenje absorbance pri 610 nm v različnih časih po opravljenem testu potrjuje stabilnost butanolnih ekstraktov resazurina. To pomeni, da lahko redukcijo resazurina izmerimo v času enega tedna po izvedbi testa, kar zvišuje njegovo uporabno vrednost. Test bi namreč lahko uporabljali tudi v terenskih pogojih, kjer spektrofotometer med izvajanjem testa ni na voljo.

Ključne besede: sperma - analize; primerjalna študija; statistika - metode; plodnostni faktorji - analize