

EVALUATION OF SULFURIC ACID, BARIUM CHLORIDE, AND SEED GERMINATION ASSAY METHODS AS EARLY PREGNANCY DETECTION INSTRUMENTS IN CATTLE

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ABSTRACT

This study aims to validate and re-evaluate the use of H₂SO₄, BaCl₂, and seed germination assay (SGA) methods to detect early pregnancy in cattle. Validation was carried out in order to find out the actual test performance value, which include accuracy, sensitivity, and specificity. Validation test was done by reacting the urine of pregnant and non-pregnant cattle populations with H₂SO₄ solution diluted at a ratio of 1 : 4, with 1% BaCl₂ solution or through germination of mung bean seeds in Petri dishes. The results of this study indicated that the H₂SO₄ and BaCl₂ methods have bad accuracy (21.88% and 28.13%) with the sensitivity of 81.82% and 100% and specificity of 9.43% and 13.21%, respectively. Meanwhile, the SGA method had a sufficient accuracy (74.55%) with sensitivity and specificity of 45.46% and 81.82%, respectively. Based on these results, the H₂SO₄, BaCl₂, and SGA methods are not recommended for detection of early pregnancy in the field.

Key words: early pregnancy detection, barium chloride, cattle, germination, sulfuric acid

ABSTRAK

Penelitian ini bertujuan memvalidasi dan mengevaluasi ulang penggunaan metode H₂SO₄, BaCl₂, dan perkecambahan biji kacang hijau (SGA) untuk mendeteksi kebuntingan dini. Validasi dilakukan agar diketahui nilai performa uji yang sebenarnya, yakni berupa akurasi, sensitivitas, dan spesifisitasnya. Validasi dilakukan dengan menguji urine populasi sapi yang bunting dan tidak bunting dengan cara mereaksikannya dengan larutan H₂SO₄ yang diencerkan 1 : 4, larutan BaCl₂ 1%, dan melakukan SGA di cawan Petri. Hasil penelitian menunjukkan bahwa metode H₂SO₄ dan BaCl₂ memiliki akurasi yang buruk (21,88% dan 28,13%) dengan sensitivitas masing-masing uji berada pada nilai 81,82% dan 100% serta spesifisitasnya 9,43% dan 13,21%. Metode SGA memiliki akurasi yang cukup baik (74,55%) dengan sensitivitas dan spesifisitasnya masing-masing sebesar 45,46% dan 81,82%. Berdasarkan hasil tersebut metode H₂SO₄, BaCl₂, dan SGA tidak direkomendasikan untuk digunakan di lapangan.

Kata kunci: deteksi kebuntingan dini, barium klorida, sapi, perkecambahan, asam sulfat

INTRODUCTION

Early pregnancy detection is a very important requirement in livestock reproduction management because it can shorten the breeding distance. Typically, pregnancy is detected using rectal palpation, an examination requires high skills but risks causing miscarriage in early pregnancy (1-2 months). Rectal palpation will only be efficient if done on cattle between day 45 and 60 gestation (Karen *et al.*, 2011). Besides, this technique can be very tiring if the examination is carried out on large numbers of animals. Another pregnancy detection technique is ultrasonography (USG). This technique is capable of detecting early pregnancy since the age of 20 days (Curran *et al.*, 1986; Kastelic *et al.*, 1989). However, the price of USG machine is relatively expensive and may not be used for examination in large numbers of animals.

There are reports of early pregnancy detection with a simple method such as by using sulfuric acid (H₂SO₄) (Sayuti *et al.*, 2011; Illawati, 2014; Fathan *et al.*, 2018), barium chloride (BaCl₂) (Lalrintluanga and Dutta, 2009), and seed germination assay (SGA) (Swamy *et al.*, 2010; Rine *et al.*, 2014; Aswathnarayanappa *et al.*, 2019). Some researchers have claimed that the H₂SO₄ method has high diagnostic accuracy, namely 94% (Hardiyanto, 2018) and 96.96% (Illawati, 2014). The

performance of evaluation of the test technically still invites a lot of criticism related to the validation method.

Evaluation of diagnostic test kit performance is accomplished by validating its test using samples that are known to be positive (pregnant) and negative (non-pregnant). In the validation of a diagnostic test kit, there must be at least a number of diagnostic variables such as sensitivity, specificity, accuracy, area under curve (AUC), positive predictive value (PPV), negative predictive value (NPV), and clinical use index (CUI) value (Subekti and Yuniarto, 2020). But, these early pregnancy detection test kit are not able to show diagnostic variables adequately. Therefore, it is necessary to validate and re-evaluate the use of these methods as an instrument for early pregnancy detection through urine samples. It is accomplished in order to know actual performance of these methods for early pregnancy detection in the field precisely.

MATERIALS AND METHODS

Sample and Study Location

The samples used in this study were the urines collected in the morning from 120 of female Friesian Holstein (FH) from Lembang, West Bandung Regency and Cipelang, Bogor Regency whose status was known to be not pregnant and had been artificially

inseminated. After selection and confirmation of pregnancy status based on rectal palpation and official records, only 64 cattle could be used as true positive and true negative standard samples. The samples were then used for early pregnancy detection experiment.

Early Pregnancy Detection Using H₂SO₄ and BaCl₂ Methods

The H₂SO₄ method was done using the modified method described by Illawati (2014). Briefly, absolute H₂SO₄ was diluted 1 : 4 with distilled water. Urine samples were mixed with the diluted H₂SO₄ with a volume ratio of 1 : 2 (based on optimization results). Samples from pregnant animal results in changes in the color of the mixture.

In testing using the BaCl₂ method (Lalrintluanga and Dutta, 2009) the urine sample was diluted 1 : 1 with 1% BaCl₂ solution. Samples from pregnant animal will result in clear solution and those from non-pregnant animals will result in the appearance of precipitation in the solution and the color change of solution into a turbid.

Early Pregnancy Detection Using Seed Germination Assay (SGA) Method

The principle of the SGA experiment conducted in this study was to germinate mung bean seeds in a Petri dish following the method described by Rine *et al.* (2014). Only 55 of 64 standard samples were used in this method. Urine samples were diluted 1 : 2 in distilled water and homogenized. The mixture (15 mL) was poured into a cotton contained Petri dish (diameter = 10 cm). A total of 20 mung bean seeds were then placed onto the wetted cotton. The Petri dish was then covered and incubated in a dark room at room

temperature for three days and observed. The percentage of mung bean seeds germinated was calculated.

Data Analysis

The data obtained were then analyzed conventionally using the Diagnostic Test (2x2 contingency table) MedCalc[®] Ver. 19 software (Subekti and Yuniarto, 2020) and Clinical Utility Index Calculator (Mitchell, 2011).

RESULTS AND DISCUSSION

H₂SO₄ Method

From the test using the H₂SO₄ method, it was found that 81.82% (9/11) of pregnant cows were detected positive (Figures 1-A and B). The contrasting results appeared in the ability of this method to detect not pregnant cows, showing only 9.43% (5/53) subjects were detected negative, and 90.57% (48/53) samples tested positive (false positive). Thus, pregnancy test using H₂SO₄ method tends to give a false positive result.

Overall, the use of the H₂SO₄ method for early pregnancy detection in cattle only had an diagnostic accuracy, sensitivity and specificity rates of 21.88%, 81.82% and 9.43%, respectively. The AUC value of the method was only 0.46 (Table 1), meaning that this test method is categorized as not useful to be applied. This finding contradicts to the reports by Cinagara-Bogor's Animal Health Training Center (BBPKH) that state the accuracy of the H₂SO₄ method was 94% (Hardiyanto, 2018) and 96.96% (Illawati, 2014).

The claim of high accuracy by the Cinagara BBPKH might be related to the mistake in determining

Table 1. The performance of H₂SO₄, BaCl₂ and SGA methods as early pregnancy instruments

| | H ₂ SO ₄ | BaCl ₂ | SGA |
|--------------------------------------|--------------------------------|--------------------------|--------------------------|
| Sensitivity (%) | 81.82 (48.22 – 97.72) | 100 (71.51 - 100) | 45.46 (16.75 – 76.62) |
| Specificity (%) | 9.43 (3.14 – 20.66) | 13.21 (5.48 – 25.34) | 81.82 (67.29 – 91.81) |
| Accuracy (%) | 21.88 (11.74 – 32) | 28.13 (17.11 – 39.14) | 74.55 (63.03 – 86.06) |
| PPV (%) | 15.79 (12.28 – 20.07) | 19.29 (17.72- 20.99) | 38.46 (20.24 – 60.61) |
| NPV (%) | 71.43 (35.68 – 91.85) | 100 | 85.71 (77.46 – 91.29) |
| + LR | 0.903 (0.68 – 1.21) | 1.15 (1.04 – 1.28) | 2.50 (1.02 – 6.16) |
| - LR | 1.93 (0.43 – 8.69) | 0 | 0.67 (0.38 – 1.16) |
| AUC | 0.46 (0.33 – 0.59) | 0.57 (0.44 – 0.69) | 0.64 (0.49 – 0.76) |
| Diagnostic Accuracy Classification * | Test Not Useful | Bad | Sufficient |
| Clinical Utility (+Ve) | 0.13 (0.00 – 0.34) | 0.19 (0.00 – 0.39) | 0.175 (0.00 – 0.52) |
| Classification ** | Very Poor | Very Poor | Very Poor |
| Clinical Utility (-Ve) | 0.07 (0.00 – 0.26) | 0.13 (0.00 – 0.33) | 0.70 (0.61 – 0.79) |
| Classification ** | Very Poor | Very Poor | Good |

PPV= positive predictive value, NPV = negative predictive value, AUC = area under curve, + LR = positive likelihood ratio, - LR = negative likelihood ratio * = Based on criteria established by Šimundić (2009), ** = Based on criteria established by Mitchell (2011)

test accuracy. Illawati (2014) stated that the ability of H_2SO_4 and rectal palpation methods in detecting pregnancy in artificially inseminated cows was 80% and 82.5%, respectively. The 80% value in the previous report is actually the sensitivity value of H_2SO_4 method that is in this study found around 81.82%. Moreover, calculation of test accuracy in the previous report was obtained from the quotient between the sensitivity of

the H_2SO_4 method (80%) and the sensitivity of the rectal palpation method (82.5%). Mathematically, this is a serious error because the formula in calculating the test accuracy is the ratio between total number of positive samples detected positive and total number of negative samples detected negative with the total sample tested (Eusebi, 2013).

Sensitivity indicates the ability of a diagnostic kit in

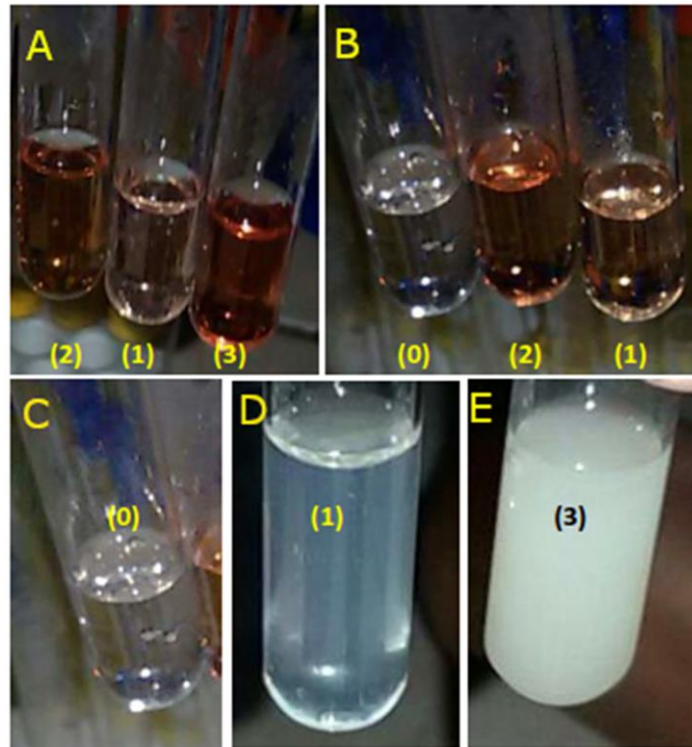


Figure 1. Results of early pregnancy detection sulfuric acid and barium chloride methods. A and B= Sulfuric acid method, C, D, and E= Barium chloride method, 0= Indicates no reaction in the test, 1-3= Indicate the degree of the reaction shown from lower to higher. Figures 1-3 indicate cows are pregnant in the H_2SO_4 and $BaCl_2$ methods.

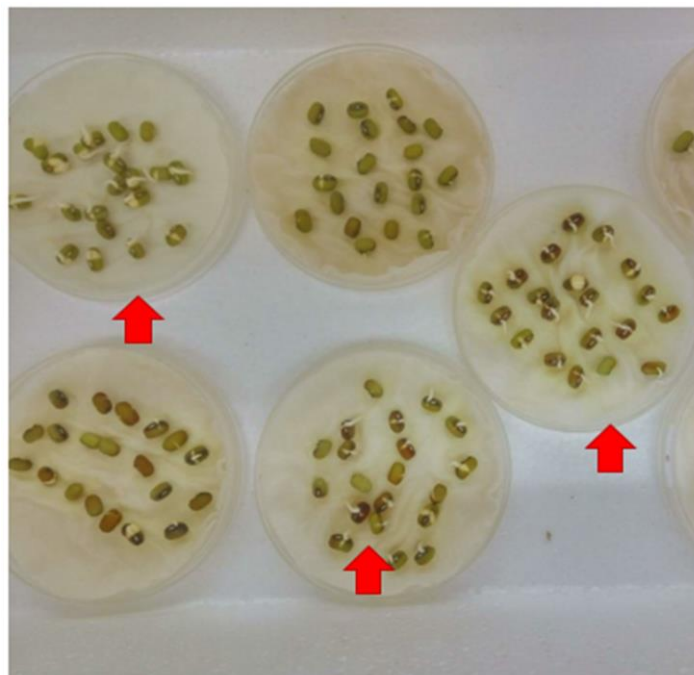


Figure 2. Results of early pregnancy detection by the SGA method. Samples that have mung bean germination are shown by red arrows.

establishing positive status in truly pregnant animals. Specificity indicates the ability of a diagnostic kit in determining negative status in truly non-pregnant animals. Test accuracy is the ability of a diagnostic kit in establishing positive status in individuals who are truly pregnant and negative status in individuals who are truly not pregnant.

Therefore, the statement that early pregnancy detection by the H₂SO₄ method has an accuracy of 96.96% is an error that scientifically unacceptable, either mathematically or by definition of a diagnostic test. The same thing was done by Sayuti *et al.* (2011) who reported that the H₂SO₄ method test accuracy was 75% in the first month. The value actually does not indicate accuracy, rather sensitivity because it is obtained from the positive detection results artificially inseminated animals. Generally, all errors in determining the test accuracy of a method are caused by errors in understanding and calculating accuracy, sensitivity, specificity, and exclusion of non-pregnant animal samples in validation. This causes information distortion on diagnosis accuracy of H₂SO₄ method in detecting pregnant cows, especially on cows whose status is not pregnant or unknown.

In general, based on the CUI (clinical utility index) value, practical use of the H₂SO₄ method for determination of pregnant animals in the field is classified as very poor. It is indicated by the low CUI +ve value (Table 1). Similarly, the ability of this method as screening test in non-pregnant animals (based on CUI -ve values) was also very poor. This is also corresponds to the accuracy criteria of diagnostic tests that stated not useful based on the AUC value.

BaCl₂ Method

In general, pregnant cow urine will remain clear when mixed with a 1% BaCl₂ solution in a ratio of 1 : 1 (Lalrintluanga and Dutta, 2009). Conversely, the solution will turn turbid or cause precipitation when non-pregnant cow urine is mixed with 1% BaCl₂ solution (Ndu *et al.*, 2000; Lalrintluanga and Dutta, 2009). However, in this study all pregnant cow urine became turbid or raised precipitation when mixed with 1% BaCl₂ solution (Figure 1-C, D, and E). The exact cause of the difference in reaction is not yet known.

In summary, the accuracy of BaCl₂ method is only 28.13% with a sensitivity of 100% and a specificity of 13.21%. The AUC value of this method is only 0.57, and it is categorized as bad diagnostic accuracy. This method also has the same tendency as the H₂SO₄ method, which gives false positive results to animals whose status is unknown or not pregnant. This result is in contrast with the Febrianingtyas *et al.* (2018) study which reported that the BaCl₂ method had sensitivity and specificity of 40.54% and 100%, respectively, with the accuracy of the test was estimated at 46.30%. The high specificity value found in this study is probably caused by the lack of non-pregnant (true negative) cow urine sample (only four cows).

Different results were reported by Skálová *et al.* (2014), that the sensitivity and specificity of this method were 79.70% and 50%, respectively from the

40 samples tested with a 1: 1 proportion between pregnant and non-pregnant cows. Lalrintluanga and Dutta (2009) also reported that the BaCl₂ method had an inadequate sensitivity and specificity, i.e. 64% and 84%, respectively from 50 samples tested (the proportion of test samples between pregnant and non-pregnant cows was by 1: 1). Based on the report of Skálová *et al.* (2014) it is estimated that the accuracy of the BaCl₂ method is around 63.89%. Meanwhile, based on Lalrintluanga and Dutta (2009), the accuracy of the BaCl₂ method is estimated at 72.91%.

Overall, based on various research reports, early pregnancy detection using the BaCl₂ method showed inconsistent results. Therefore, the results are very inconclusive if the method is applied in the field. Our study validation showed that based on CUI values BaCl₂ method gave very poor performance, either in determining animal pregnancy or as a screening tests in non-pregnant animals (Table 1). If we traced back, the study of Skálová *et al.* (2014) and Lalrintluanga and Dutta (2009) are estimated to be in the category of poor to fair performance based on CUI scores. Thus the H₂SO₄ and BaCl₂ methods for early pregnancy detection do not yet have sufficient accuracy to be widely applied in the field.

Seed Germination Assay (SGA) Method

Five of 11 (45.46%) samples of pregnant cows were able to be detected positive by the SGA method. In contrast, 36 out of 44 (81.82%) non-pregnant cow samples were able to be detected negative (Table 1). The results of testing using the SGA method tended to provide a negative interpretation of pregnant cows. However, this test has good capability in detecting non-pregnant cow (Figure 2). In general, the SGA method test accuracy was 74.55% with diagnostic sensitivity and specificity of 45.46% and 81.82%, respectively. The AUC value of the SGA method is only 0.64 and it is categorized as having sufficient diagnostic accuracy.

In this study it was known that pregnant cows had a lower germination rate, namely $\leq 60\%$. This result is similar to the study of Rine *et al.* (2014) which stated that artificially inseminated cows had very different percentage of germination inhibition ($P < 0.01$) from cows that were not artificially inseminated, 65.31% in artificially inseminated cows and 40.60% in cows that were not artificially inseminated. A similar result was reported by Perumal (2014), Swamy *et al.* (2010), Krishna and Veena (2009), and Dilrukshi and Perera (2009) all stated significant differences ($P < 0.01$) in the germination inhibition in SGA method.

In contrast, the studies of Ghalioungui *et al.* (1963) and Kubátová and Fedorova (2016) gave different results. Ghalioungui *et al.* (1963) stated that in pregnant women, germination is more fertile than in non-pregnant women. In addition, Kubátová and Fedorova (2016) also stated that pregnant alpaca shows richer germination compared to non-pregnant one. However, none of the results of these studies describe the sensitivity, specificity, or accuracy of SGA which are the main criteria for evaluating a method or diagnostic kit, so a comprehensive analysis of this method is not found.

Thus, the validation principles of SGA method on the previous studies, in general, are still far from sufficient to be called capable of proving its performance. At least this study is able to provide an initial overview that its validation is carried out following the validation principles. Based on the results of the validation in Table 1, it is known that the SGA method can only be applied as a screening test in determining of non-pregnant cows. This is based on the CUI -ve value which is categorized as good and the AUC value which categorizes the diagnostic accuracy of the SGA method as sufficient. However, given its average specificity and NPV was 81.82% and 85.71%, respectively, the ability of this method to detect non-pregnant cows was only 70.12%. On the other hand, germination variables to detect early pregnancy is vary between regions—sometimes germination is seen in the urine of pregnant cows in one region and vice versa in another region (unpublished data). Therefore, this method is location-specific and species-specific like the various results reported by other researchers.

CONCLUSION

The H₂SO₄, BaCl₂, and SGA methods are considered inadequate for use as an early pregnancy detection instruments in cattle with a test accuracy of 21.88% with an AUC of 0.46 (test not useful), 28.13% with an AUC of 0.57 (bad diagnostic accuracy) and 74.55% with an AUC of 0.64 (sufficient diagnostic accuracy), respectively.

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