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Research article

Barium chlorideassay of urine: A cost-effective method for early diagnosis of pregnancy in cow

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ABSTRACT

To reduce calving interval through an appropriate reproductive management program, the early detection of pregnancy at the 1st trimester is essential to enable the prompt re-insemination of non-gravid cows. We aimed to re-evaluate the efficacy of barium chloride assay to detect early pregnancy in cross-bred cows. Urine and blood samples were collected from 72 and 65 artificially inseminated cows, respectively. Pregnancy status at 1st (Day: 1-90) and 2nd (Day: 91-180)trimesters were compared using two different methods: a barium chloride assay from urine samples and a progesterone enzyme linked immune sorbent assay (P_4-ELISA) from blood samples, where rectal palpation was considered as gold-standard. We found that although the overall (combining both 1st and 2nd trimesters) true positive, negative predictive value (NPV) and accuracy of P_4-ELISA was better, the barium chloride assay performed equally well P_4-ELISA in terms of true positive percentage of pregnancy diagnosis (70.8% in barium chloride vs. 85.7% in P_4-ELISA; P>0.05) at the 1sttrimester. In addition, specificity of pregnancy diagnosis was higher in barium chloride test than P_4-ELISA at the 1sttrimester (100% in barium chloride vs. 66.7% in P_4-ELISA). Therefore, barium chloride method could be a good choice under farm and field set up of early pregnancy diagnosis at the 1sttrimester in cows.

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1.INTRODUCTION

Pregnancy diagnosis is an essential aspect for successful breeding program in a cattle farm. Pregnancy detection at the early stage of pregnancy is vital for reducing the calving interval through enabling the farmers to address the reproductive disorders and/or re-breed shorty after conception failure. In a successful breeding program, to get one calve per year, ideally post-partum period should not exceed85-days (El-Rheem et al., 2022). Therefore, the dairy farmers need to identify non-pregnant cows at the earliest time so that they can rebreed them at the subsequent oestrous.

In cow, the 1st 42 days after a successful insemination is considered as early embryonic period (Peippo et al., 2011). During this time, fertilization and implantation take place. A successful implantation is usually maintained until calving giving provided that there is no

infectious disease, or injuryintervenes the survival of embryo and fetus (Morris and Disk in 2008). For the efficient diagnosis of pregnancy, the ideal test should have high sensitivity (able of identify pregnancy correctly) and specificity (able to identify non-pregnancy accurately). Moreover, the test should be cheap and simple enough to apply in the farm condition. In addition, it would be useful if the test provides more information such as viability and/or gender of embryo and foetus. In broader aspect, there are two categories of pregnancy diagnosis test: direct and indirect methods. In the direct methods, the pregnancy is diagnosed by the operator's hand feeling during rectal palpation or observation of ultrasound image (Mezianet al., 2021). Both of these methods are sensitive and specific enough to certainly identify pregnancy, however skilled manpower is required for their application. In direct method includes identifying pregnancy-associated biomolecules in blood or urine. For early detection of pregnancy in cow, several pregnancy-associated biomolecules studied from the blood and urine samples. These includes early pregnancy factor (EPF), progesterone (P_4), pregnancy associated glycoprotein (PAGs), interferon-stimulated genes (ISGs) etc. (Balharaet al., 2013). None of these marker-based methods are efficient enough to identify pregnancy correctly. Therefore, farmers are mainly relying on rectal palpation and ultrasound imaging for pregnancy diagnosis.

Since the pregnancy detection by rectal palpation or ultrasound requires highly skilled personnel and marker-based assays are inaccurate and costly, most of the farmers in in low-income countries cannot afford them, leading to delays in detecting pregnancyresulting in reduced productivity. Thus, there is a crucial need for a low-cost, user-friendly method of pregnancy diagnosis that is suitable for the farming context of low-income countries. In this study, we re-evaluated the efficiency of barium chloride method of pregnancy diagnosis in cow. In this method, diluted barium chloride is added to the urine of the cow under investigation and the non-pregnancy is interpreted by the formation of white precipitates of barium chloride salt. Although the precise mechanism is unknown, it is believed that barium chloride reacts with oestrogen to form precipitates and high oestrogen level in urine indicates the non-pregnancy status. A plethora of studies have been conducted to demonstrate the efficacy of barium chloride method of pregnancy diagnosis in cow. However, almost in all cases this method showed limited specificity and sensitivity in pregnancy diagnosis. In this study, we specifically looked on the 2 separate trimesters ($1^{st}$and 2^{nd}) to understand the efficacy variation of barium chloride in the diagnosis of pregnancy. Interestingly, we found that as compared to progesterone-ELISA (P_4-ELISA), barium chloride is performed equally and better respectively in terms of accuracy and specificity for the diagnosis of pregnancy in the 1^{st}trimester. Therefore, we concluded that barium chloride method of pregnancy diagnosis could be an alternative tocurrent practicing methods of pregnancy diagnosis where facilities are limited. Under filed and farm set up, where modern facilities are limited, this method could be useful and cost effective to identify pregnancy at early stages and thus attributing to calving interval.

2.MATERIALS AND METHODS

Ethics and consent to participate

The experiments were conducted as per the guidelines of the ethical committee of Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. The animals in this study were owned by a farm owner. Therefore, informed consent was taken before the animals were included in this study.

Animals and samples

Urine and blood samples from pregnant cows were collected during the 1^{st} trimester (Days 1–90) and the 2^{nd} trimester (Days 91–180) of pregnancy. A total of 72 urine (24 and 48 respectively in the $1^{st}$and 2^{nd}trimesters) and 65 blood (28 and 37 respectively in the $1^{st}$and 2^{nd}trimesters) samples were collected from pregnant cross-bred dairy cows from a private commercial dairy farm of Chattogram city. Animals were selected based on insemination records and insemination dates were noted accordingly from the farm record sheet. Blood and urine samples were collected from these animals at their first (4^{th}week, 8^{th} week and

12th week of pregnancy) and second (16th week, 20th week and 24th week of pregnancy) trimesters. Around 300-500 mL of morning (8.00 A.M. to 10.00 A.M.) urine sample was collected from each selected animal directly from the stream using a clean glass beaker while animal was urinating. Five mL of blood was collected from tail vein and transferred to vacutainer without having anticoagulant. The urine samples were used for an on-spot barium chloride test. Blood samples were kept in ice and transferred to the laboratory within 3 hours of collection for serum separation and storage. The clotted blood samples were centrifuged at 1500×g for 10 minutes for serum separation, which was then stored at -20ºC until analysis for serum progesterone level.

Diagnosis of pregnancy

Rectal palpation method

The pregnancy was confirmed by rectal palpation at 12 weeks of pregnancy, and it was considered gold-standard for pregnancy diagnosis in this study. To avoid false positive pregnancy by various pathological structures such as cyst, tumor etc., and the animals were monitored until parturition, and only those that had a successful parturition were considered in this study.

Chemical-based method (by barium chloride)

For the detection of pregnancy by chemical method, barium chloride was used (Haque et al., 2023). Briefly, 5 mL of freshly drawn urine was taken into a test tube. Then, 5-6 drops of 1% barium chloride were added into the urine sample followed by gently mixed and incubated at environmental temperature (varied from 25-32ºC) for 5 minutes. An opaque precipitation after incubation indicated the non-pregnant cow whereas pregnancy indicator is the lack of precipitation (Figure 1). Although it is unclear which component of urine of pregnant cow prevent the formation of barium chloride precipitation (Kubátová et al., 2016), it is thought that barium chloride reacts with estrogen to form the precipitates (Haque et al., 2023) and/or the metabolic product of progesterone inhibits the precipitates formation. Progesterone is converted by the liver to pregnanediol glucosiduronidate, an inactive metabolite, which is eliminated through urine (Schiffer et al., 2019). Urine samples can be utilized in the barium chloride test to identify this progesterone residue (Kubátová et al., 2016). Since the level of estrogen drastically reduces and progesterone metabolites increases in pregnant cows, barium chloride fails to form precipitates.

Figure 1. Pregnancy diagnosis by barium chloride assay from urine sample. After incubation of urine with barium chloride for 5 minutes, an opaque precipitation (test tube 1) indicates non-pregnant cow whereas a clear urine (Test tube 2) indicates pregnancy.

Measuring progesterone levels (by P4-ELISA)

The separated serum samples were used to measure the progesterone levels by an antigen-captured ELISA method using a commercially available Progesterone AccuBind ELISA Kit (#4825300, Monobind Inc.(R), California) with the manufacturer's protocol. The absorbance for ELISA was recorded using a Microplate reader with a filter of 450 nm wavelength (reference wavelength 620 nm). The absorbances were then fitted to standard carve generated from known amounts of progesterone and the progesterone levels in serum were determined

by regression equation. A progesterone level>4 ng/mL in the serum in any time point ($1^{st}/2^{nd}$trimester) of analysis was considered as successful pregnancy by the artificial insemination.

Data analysis

The hormonal data of progesterone (>4ng/mL) and urinary barium chloride data were analyzed to check if chemical-based result could predict early pregnancy. The results were sub-divided as: True negative (TN), True positive (TP), False negative (FN) and False-positive (FP). From these values, the accuracy, sensitivity, specificity along with, positive and negative predictive values were calculated based on the formulae: Accuracy =100 × ((TP + TN) ÷ (TN + TP + FP +FN)), Sensitivity = 100 × (TP ÷ (TP + FN), Specificity= 100 × (TN ÷ (TN+ FP), The positive predictive value (PPV)= 100 × (TP ÷ (TP+ FP) and the negative predictive value (NPV) = 100 × (TN ÷ (TN+ FN).

3. RESULTS

In this study, the outputs from barium chloride and P_4-ELISA test were grouped into different categories according to accuracy of pregnancy diagnosis compared with rectal palpation, which is considered as gold-standard. Between the test methods, P_4-ELISA was significantly (P<0.001) better in true positive percentages of pregnancy detection. P_4-ELISA detected 83.1% cows as true pregnancy-positive whereas barium chloride test could detect only 40.3% as true pregnancy-positive (Table 1).

To understand the efficacy of these two tests in different trimesters of pregnancy the result outputs were grouped for 1^{st} and 2^{nd} trimesters. Notably, there were insignificant (P>0.05) differences in true positive percentage of pregnancy detection in the 1^{st} trimester between methods (70.8% in barium chloride vs. 85.7% in P_4-ELISA). However, we observed that P_4-ELISA test performed better (P<0.01) in terms of accurate detection of pregnancy (27.1% in barium chloride vs. 86.4% in P_4-ELISA) in the 2^{nd} trimester (Table 1).

The overall comparative performance parameters of the barium chloride and P_4-ELISA test are presented in Table 2. In all cases, the overall performance parameters to detect pregnancy viz. sensitivity (63% in barium chloride vs. 96.4% in P_4-ELISA), PPV (87.8% in bairam chloride vs. 98.1% in P_4-ELISA), NPV (56.4% in barium chloride vs. 80% in P_4-ELISA) and accuracy (70.8% in barium chloride vs. 95.3% in P_4-ELISA) are higher in P_4-ELISA except for the case of specificity where both test methods performed equally.

Table 1.Categories of data obtained from pregnancy detection of cows by barium chloride and P_4-ELISA test (rectal palpation: Gold-standard)

Test	**TP, n (%)**	**TN, n (%)**	**FP, n (%)**	**FN, n (%)**	**Total, n (%)**
Overall (Combining 1^{st} and 2^{nd} trimester)					
Barium chloride	29 (40.3)	22 (30.5)	4 (5.6)	17 (23.6)	72 (100)
P_4-ELISA	54 (83.1)	8 (12.3)	1 (1.5)	2 (3.1)	65 (100)
P value	<0.001	0.001	<0.21	<0.001	
1^{st} trimester					
Test	**TP, n (%)**	**TN, n (%)**	**FP, n (%)**	**FN, n (%)**	**Total, n (%)**
Barium chloride	17 (70.8)	3 (12.5)	0 (0)	4 (16.7)	24 (100)
P_4-ELISA	24 (85.7)	2 (7.1)	1 (3.6)	1 (3.6)	28 (100)
P value	0.19	0.51	0.34	0.11	
2^{nd} trimester					
Barium chloride	13 (27.1)	18 (37.5)	4 (8.33)	13 (27.1)	48 (100)
P_4-ELISA	32 (86.4)	3 (8.1)	2 (5.4)	2 (5.4)	37 (100)
P value	<0.001	0.002	0.60	0.009	

1^{st} trimester: Day 1-90, 2^{nd} trimester: Day 91-180,TP: True positive, TN: True negative, FP: False-positive and FN: False negative. P valuesare obtained from $\chi 2$ test.

These findings suggest that the P_4-ELISA would be more useful compared to barium chloride test for pregnancy diagnosis of cows. To understand the efficacy of these tests during 1st and 2nd trimester of pregnancy, the performance parameters also compared in these two phases (Table 2). Interestingly this comparison demonstrated a range of interpretations. During the 1st trimester, the barium chloride method was less sensitive (80.9%) but more specific (100%) compared to P4-ELISA, which had a sensitivity of 96% and a specificity of 66.7%, for pregnancy diagnosis. In addition, PPV was higher for barium chloride test (100% in barium chloride vs. 96% in P_4-ELISA). However, NPV (42.8% in barium chloride vs. 66.7% in P_4-ELISA) and accuracy (83.3% in barium chloride vs. 92.8% in P_4-ELISA) were lower in barium chloride test as compared to P_4-ELISA. In the 2nd trimester, although having lower specificity (81.8% in barium chloride vs. 60% in P_4-ELISA) than barium chloride the P_4-ELISA performed better for its sensitivity (50% in barium chloride vs. 94.1% in P_4-ELISA), PPV (76.4% in barium chloride vs. 94.1% in P_4-ELISA), NPV (58.0% in barium chloride vs. 60.0% in P_4-ELISA) and accuracy (64.5% in barium chloride vs. 94.6% in P_4-ELISA), respectively. Based on these findings, for its higher true positive percentages, specificity and PPV barium chloride test be considered as a useful alternative test for the diagnosis of pregnancy at the 1st trimester whereas P_4-ELISA would be more suitable in the 2nd trimester.

4. DISCUSSION

A shorter calving interval increases profitability of dairy farms and without a doubt, early identification of pregnancy is critical for reducing the calving interval. Many researches have focused on detecting pregnancy using ELISA and radioimmunoassay (RIA) techniques to determine progesterone levels in blood, milk, and fecal samples (Samsonova*et al.*, 2014). For the results to be read, these techniques require expensive reagents and sophisticated laboratories which are almost impossible to adopt in the farming conditions of low-income countries. In addition, due to the requirement of radioactive isotopes, RIA is highly hazardous. The direct method, rectal palpation and ultrasound imaging are not also affordable in farming condition of many countries due to unavailability of skilled manpower. On the other hand, the barium chloride test is a simple and easy technique, relatively more accessible and cost effective to be adopted at farm conditions.

The hormone progesterone, secreted by the placenta during pregnancy, is metabolized in the liver to an inactive compound called pregnanediol glucosiduronidate, which is eliminated in urine (Schiffer et al., 2019). Urine samples can be utilized in the barium chloride test to identify this progesterone residue (Kubátová et al., 2016) or high level of estrogen. As a reference (gold) standard, for very early detection of pregnancy, the uterus of slaughtered cow can be observed under microscope. Because of high accuracy rate (99.99%) over the years, researchers utilized it as a benchmark to justify alternative methods of pregnancy diagnosis (Neto et al., 2010). In our research, we used rectal palpation, which is a popular direct method of pregnancy diagnosis, as the reference. The accuracy of other methods in pregnancy diagnosis is variable. For example, accuracy of ultrasound imaging at 27 days of pregnancy is about 93.7% to 97.8% (Silva et al., 2007). In the current study, the sensitivity of the P_4-ELISA for pregnancy diagnosis was 96.4%. This finding is similar to that of (Inaudi *et al.*, 1982), who discovered 93.5% sensitivity for pregnancy diagnosis. In contrast, researchers found 84.8% sensitivity, which was less than the results of this study. This could be the result of presence of continuous corpus luteum (CL) or quiet heat, or as a result of an early embryonic demise (Ghaidan et al., 2019). In this study, we observed 63.04% sensitivity 84.6% specificity in pregnancy diagnosis for barium chloride test. On the other hand, the specificity of P_4-ELISA, was 88.9%. The relatively low specificity of barium chloride test might be due to the fact that the CL secretes P_4, which is found in both pregnant and non-pregnant cows (Jaimes et al., 2019). As a result, P_4 is not just for pregnant animals. The progesterone ELISA's accuracy in detecting pregnancy was 95.3%. Our results are higher than those of Samsonova et al.(2014), who reported 87.0%, and Ghaidan et al.(2019). After artificial insemination, accuracy rates increased from 85.4% to 88.4%, according to Pennington et al.(1985). The technique used to diagnose pregnancy has an impact on accuracy;

ELISA has an accuracy of about 95.3%, whereas RIA has an accuracy of about 80.5% (Nebel et al., 1987). In the current investigation, the barium chloride test was 70.8% accurate in detecting pregnancy. This discrepancy in accuracy might be caused by a persistent CL, which raises false-positive pregnancy diagnosis outcomes. Furthermore, excessive feed intake by cows stimulates progesterone metabolism (Vasconcelos et al., 2003), resulting in higher pregnanediol glucosiduronidate concentrations in the urine and more false positive results. Exogenous sources of estrogens in the food, in addition to this metabolite, can cause false-positive diagnosis. Low P_4 production during pregnancy, on the other hand, will increase the number of false-negative outcomes (Jaimes et al., 2019). In this study, we observed that barium chloride test performed equal or even better than P_4-ELISA at the 1st trimester of pregnancy. Therefore, the barium chloride test could be used as a better accessible alternative at field and farm set upfor pregnancy diagnosis at the 1st trimesterin case of limited and hindering resources.

Table 2.Performance parameters of barium chloride and P_4-ELISA tests for detection of pregnancy in cows

Test criteria	**Barium chloride test (N=72)**		**P_4-ELISA test (N=65)**	
	%	**95% Confidence Interval**	**%**	**95% Confidence Interval**
Overall (Combining 1st and 2nd trimester)				
Sensitivity	63.0	53.2-72.9	96.4	94.4-98.4
Specificity	84.6	76.5-92.7	88.9	82.3-92.1
PPV	87.8	81.6-91.8	98.1	95.6-99.0
NPV	56.4	48.3-64.7	80.0	72.2-87.8
Accuracy	70.8	61.9-79.6	95.3	89.9-97.1
1st trimester				
Test criteria	**Barium chloride test (N=24)**		**P_4-ELISA test (N=28)**	
	%	**95% Confidence Interval**	**%**	**95% Confidence Interval**
Sensitivity	80.9	65.3-96.4	96.0	86.3-1.0
Specificity	100	-	66.7	49.8-83.6
PPV	100	-	96.0	86.3-1.0
NPV	42.8	25.7-59.9	66.7	49.8-83.6
Accuracy	83.3	67.3-1.0	92.8	81.6-1.0
2nd trimester				
Test criteria	**Barium chloride test (N=48)**		**P_4-ELISA test (N=37)**	
	%	**95% Confidence Interval**	**%**	**95% Confidence Interval**
Sensitivity	50.0	38.6-61.4	94.1	83.7-1.0
Specificity	81.8	71.0-92.6	60.0	45.8-74.2
PPV	76.4	65.1-87.7	94.1	83.7-1.0
NPV	58.0	46.3-69.7)	60.0	45.8-74.2
Accuracy	64.5	53.0-76.0	94.6	84.4-1.0

1st trimester: Day 1-90, 2nd trimester: Day 91-180, PPV: Positive predictive value, NPV: Negative predictive value.

5. CONCLUSION

In this study we compared the efficacy of barium chloride test with P4-ELISA for pregnancy diagnosis in cow where rectal palpation method was regarded as gold standard. Considering all timepoints of pregnancy diagnosis, barium chloride had a poor performance for detecting true positive (40.3%) compared to that of P4-ELISA (82.1%).However, in the case of early pregnancy diagnosis (1st trimester), both tests showed no significant difference in dignostic accuracy. Moreover, barium chloride test was more specific. Since barium chloride test is cheap and handy to conduct, it can be used an effective alternative to P4-ELISA or rectal palpation, where more expenses or skills, for early pregnancy diagnosis in cow.

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