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SHORT COMMUNICATION

Punyakoti Test: A Seed Germination Inhibition Test for Early Pregnancy Diagnosis in Graded Murrah Buffaloes

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ABSTRACT

A simple non- invasive technique which was developed on the basis of ancient Egyptian knowledge called as Punyakoti test (Veena, 1997) was used to detect early pregnancy at day 26 post insemination. The present study was carried out to evaluate the accuracy of Punyakoti test, a seed germination inhibition test for early pregnancy diagnosis in graded Murrah buffaloes. The urine samples were collected from 20 buffaloes early in the morning on day 26 post insemination and were subjected to pregnancy diagnosis using this test. Urine was diluted with distilled water in sterile petri dish in the ratio of 1:14 and 15 good quality wheat seeds were added to each petri dish. A significant inhibition of seed germination after 48hrs and shoot length after 5 days was taken as criteria for declaring true pregnancy and confirmation of pregnancy was done by rectal palpation on days 45-60 post insemination. A significant difference ($P<0.05$) was noticed between pregnant and non-pregnant buffaloes regarding germination inhibition percentage and shoot length (cm) which was recorded as 70.66 ±1.63; 3.33 ± 0.074 and 45.99 ± 1.84 ; 5.44 ± 0.17, respectively. This test was 66.66 per cent accurate in diagnosing pregnancy but 90.90 per cent accurate in diagnosing non-pregnant animals.

Keywords: Murrah buffaloes, early pregnancy, accuracy, punyakoti, germination inhibition

Early diagnosis of pregnancy is an important aspect for both the reproductive management and the profitability of dairy herds. Lack of reliable cow side early pregnancy diagnostic methods has been drawing the attention of several researchers. Even though the techniques such as rectal palpation, radiography, ultrasound, progesterone assay and rosette inhibition assay are available (Jainudeen and Hafez, 1993; Wani *et al.* 2003) due to practical constraints in their application, there is a consistent effort in search of a simple, economical and non invasive technique. Punyakoti test, a simple, non invasive test has been developed to diagnose pregnancy in cattle based on the high concentration of Abscissic acid in pregnant animal urine, which causes seed germination inhibition (Veena and Narendranath, 1993). Therefore this seed germination inhibition test was undertaken for the present study to recognize it as a door step technology to the farmers as it requires inexpensive materials and does not need special skills.

MATERIALS AND METHODS

Twenty Graded Murrah she buffaloes weighing 400-500 kg aged 4-5 years presented to Teaching Veterinary Clinical Complex, Gannavaram were utilized in the present study. All the experimental animals showing estrus signs were bred by artificial insemination at 8-12 hrs after the onset of estrus using frozen semen. Pregnancy diagnosis was carried out using punyakoti test on day 26 post insemination.

Collection of Urine

The urine samples (n=20) from the experimental group animals were collected during natural micturition as well as by induced micturition, done by continuous stroking of the skin just below the vulva (Rine *et.al.*, 2014) on day 26 post insemination. The urine samples were collected early in the morning in clean, sterilized and dry plastic containers.

Test Procedure

Seed germination inhibition test was carried out according to the standard procedure (Veena, 2006) with some modifications. About 15 seeds of wheat were placed in a petri dish containing filter paper added with 15 ml of diluted urine in the ratio of 1:14 (1ml urine + 14 ml water). Similarly, a petri dish containing seeds treated with 15 ml water was kept as control and diluted urine samples from non-pregnant animals were kept as negative group. Inhibition of seed germination and discoloration of seeds after 48 hrs and reduced shoot growth after five days were taken as positive criteria for declaring pregnancy. Germination inhibition percentage and shoot length of control, negative and test groups were calculated and compared. All the experimental animals that had undergone this technique were screened per rectally on days 45-60 for confirmation of pregnancy. Germination inhibition percentage was calculated as per formula:

Germination Inhibition Percentage

It was defined as the number seeds not germinated out of the total number of seeds taken in the experiment.

$$GI\ \% = \frac{\text{No. of seeds not germinated in Petri dish}}{\text{Total no. of seeds taken in Petri dish}} \times 100$$

Accuracy in the form of sensitivity, specificity, positive and negative predictive values of the present technique was calculated as per the formulas given by Pieterse *et.al.* (1990) and were depicted as follows:

Pregnant	Non-pregnant
Diagnosis pregnant correct (a)	Diagnosis non-pregnant correct (c)
Diagnosis pregnant incorrect (b)	Diagnosis non-pregnant incorrect (d)

Number of pregnant animals = a+d

Number of non-pregnant animals = b+c

Sensitivity = a/ (a+d) × 100

Specificity = c/(c+b) × 100

Positive predictive value = a/ (a+b) × 100

Negative predictive value = c/(c+d) × 100

Overall diagnostic accuracy = a+c/ (a+b+c+d) × 100.

RESULTS AND DISCUSSION

The germination inhibition percentage of wheat seeds in control, negative and positive groups were presented (Table. 1). Germination inhibition percentage of 66.66 or more was considered as pregnant correct by this test. The Mean ± SE values of germination inhibition percentage of control, negative and positive buffaloes were 25.33 ± 1.94, 45.99 ± 1.84 and 70.66 ± 1.63 per cent respectively (Table 1). The shoot length of wheat seeds in control, negative and positive groups were presented (Table. 1). The shoot length of less than 3.4cm was considered as true pregnant in the present study. Shoot length was less in buffaloes that were diagnosed as pregnant (Fig. 1C). The Mean ± SE values of shoot length of control, negative and positive buffaloes were 10.37 ± 0.31, 5.44 ± 0.17 3.33 ± 0.074 cm respectively (Table. 1). Out of 20 buffaloes, this test has diagnosed 7 (35%) as pregnant (Fig. 1C) and 13 (65%) as non-pregnant (Fig. 1B). Out of 7 buffaloes diagnosed pregnant by this test, 1 (14.28%) of them became non-pregnant and out of 13 animals diagnosed non-pregnant, 3 (23.07%) became pregnant upon rectal palpation at day 45-60 (Table. 2). The germination inhibition percentage was 66.66 or more while shoot length was 3.4 cm or less in buffaloes that were diagnosed as pregnant correct by this test. Sensitivity, specificity, positive and negative predictive values were presented (Table. 3). This test was 66.66 per cent accurate in diagnosing pregnancy but 90.90 per cent accurate in diagnosing non-pregnancy.

Table. 1 Mean ± SE values of germination inhibition percentage and shoot length (cm) of wheat seeds in different groups

S.No	Particulars	Control	Non-pregnant	Pregnant
1.	Germination inhibition percentage	25.331 ± 1.94^{a}	45.99 ± 1.84^{b}	70.66 ± 1.63^{c}
2.	Shoot length (cm)	10.37 ± 0.31^{d}	5.44 ± 0.17^{e}	3.33 ± 0.074^{f}

P<0.05 Means with different superscripts in a row differ significantly.

Table. 2 Confirmation of pregnancy diagnosis using Punyakoti test with rectal palpation

S.No	Diagnostic results	Punyakoti test (day 26)	Rectal palpation (d 45-60)	Diagnosis incorrect
1.	Pregnant	7 (35%)	6/7 (85.72%)	1/7 (14.28%)
2.	Non-pregnant	13 (65%)	10/13 (76.93%)	3/13 (23.07%)

Table. 3 Accuracy of pregnancy diagnosis using Punyakoti test (n=20)

S.No	Particulars No. of animals tested	Number
1.	Diagnosis pregnant correct	6
2.	Diagnosis non-pregnant incorrect	1
3.	Diagnosis non-pregnant correct	10
4.	Diagnosis non-pregnant incorrect	3
5.	Sensitivity (Se ; %) 100xa/(a+d)	66.66
6.	Specificity (Sp ; %) 100xc/(c+b)	90.90
7.	Positive predictive value (PPV ; %) 100xa/(a+b)	85.71
8.	Negative predictive value (NPV ; %) 100xc/(c+d)	76.91
9.	Overall diagnostic accuracy	75

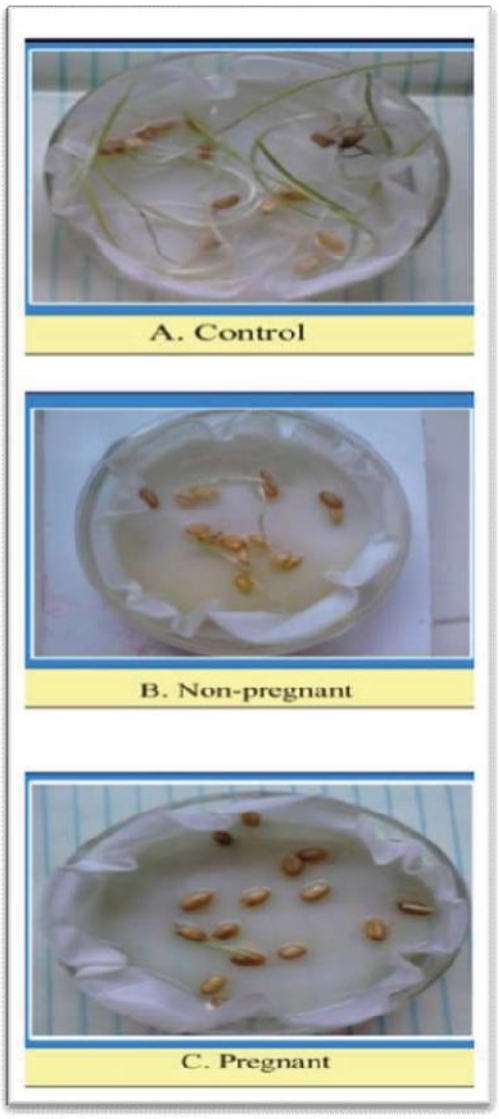

Fig. 1. Interpretation of early pregnancy using Punyakoti test

In the urine of 7 buffaloes out of 20, the seed germination inhibition was 70.66 ± 1.63 Vs 45.93 ± 1.84 per cent and the shoot length was short as 3.33 ± 0.074 Vs 5.444 ± 0.17 cm in pregnant and non-pregnant respectively which were found positive on rectal palpation at day 45-60 post insemination. The observations of this study with regards to germination inhibition per cent and shoot length in cm 70.66 ± 1.63; 3.33 ± 0.074 Vs 45.99 ± 1.84; 5.44 ± 0.17 in pregnant vs non-pregnant buffaloes were significantly different ($P<0.05$). The buffaloes which have shown more wheat seed germination inhibition (70.66) and shoot length (5.44) cm later found negative for pregnancy at day 45-60 on per rectal examination. The recorded germination inhibition percentage was higher with reduced shoot length showed significant ($P<0.05$) difference in pregnant when compared with non-pregnant Graded Murrah buffaloes.

The observations recorded in the present study were in close agreement with the findings of other researchers (Dilrukshi *et al.* 2009; Rao and Veena, 2009; Swamy *et al.* 2010; Perumal, 2014) in cattle. The findings of Dilrukshi *et al.* (2009) clearly stated that the urine of the pregnant cows significantly suppressed the seed germination (57.93 per cent) compared to non-pregnant (79.2 per cent). The recorded shoot length was 3.89 ± 3.16 cm and was significantly less when the seeds were treated with the urine samples collected from the pregnant cows and the same was 6.1 ± 3.24 cm for non-pregnant cows. These findings were similar to those observed in the present study (3.33 and 5.44 cm) for pregnant and non-pregnant buffaloes. The present findings are in total agreement with those of Swamy *et al.* (2010) and Perumal (2014) with regard to seed germination inhibition in pregnant animals (73.65 ± 2.81, 78.91 ± 2.09 per cent). The shoot length observed by these authors was 0.95 ± 0.47, 0.53 ± 0.52 cm. A significant inhibition of seed germination after

48 hours and shoot length after 4 days with wheat seeds at 20 days and 28 days post insemination was observed by Rao and Veena (2009) indicating the usefulness of this method to detect early pregnancy.

The factors that might be influencing such a differential response in urine treated seeds could be plant growth regulators such as auxins that are excreted in high concentrations in urine during pregnancy which might be causing inhibitory response to seed germination and shoot growth. Further it was opined by Veena and Narendranath (1993) that presence of increased concentrations of Abscissic acid (ABA) which was the inhibitory factor. The most probable factor influencing seed germination and shoot growth excreted in urine must have been associated with the events in the reproductive tract commencing from the time of conception till a few days postpartum because the inhibitory response of urine on seeds has been shown to persist for as long as three months postpartum (Veena and Narendranath, 1993).

Further it was confirmed that a high concentration of Abscissic acid (ABA) was found in the urine of pregnant cows (170.62 nmol/ml) as compared to urine of non-pregnant cows (74.46 nmol/ml) (Veena, 2006). By comparing the observations of earlier works with the present study, it was concluded that punyakoti test which was a simple, non-invasive and do not require any chemicals or sophisticated instruments can be used efficiently to diagnose early pregnancy between day 25 to 30 post insemination.

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