Research Article

The Utility of Punyakoti Test for Pregnancy Detection in Artificially Inseminated Dairy Cattle: The Case of Smallholder Farming in Zambia

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Abstract | Pregnancy diagnosis is a crucial on-farm strategy employed to improve performance and fertility of dairy cattle and minimize economic losses. Hence, this study was carried out to validate the Punyakoti test, a cheap and simple pregnancy diagnostic tool, for application in dairy cattle especially among smallholder farmers. The study involved four treatment groups: distilled water (DW), urine from artificially inseminated cows (AIC), non-inseminated cows (non-AIC), and non-inseminated heifers (non-AIH). Free-catch urine samples, from 18 randomly selected dairy animals, were collected on day 15, 22, 30, and 35 after insemination and used at a dilution rate of 1:4. Maize seeds were soaked in different treatment groups, followed by observation for germination and shoot length. Data were analysed descriptively to obtain the means, and inferential statistics including Kruskal-Wallis and Bonferroni tests were employed to explore significance between the means. The Eta coefficient test statistic evaluated the association between selected variables. The lowest means for germination (21.92±1.15) and shoot length (0.91±0.05) of seeds in AIC group were observed at day 35. The highest means on the same day were 84.00±1.95 and 3.05±0.14, for seed germination and shoot lengths in DW, respectively. The mean germination percentages of seeds in AIC differed significantly (P<0.05) between days after insemination; the mean values for other treatments did not differ significantly (P>0.05). The mean shoot lengths of seeds in AIC differed significantly (P<0.05) between days after insemination; no level of significance (P>0.05) was observed for other treatment groups. The association between treatment factor and seed germination on day 15 and 35 was 0.149 and 0.993, respectively; and was 0.166 and 0.876 on 15 and 35, respectively, with shoot lengths. In conclusion, Punyakoti test can be used for pregnancy detection, preferably towards month-end after insemination, in dairy cattle with seed germination parameter being sufficient to get reliable results.

Keywords | Dairy cattle, Maize, Pregnancy detection, Punyakoti test, Seed

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INTRODUCTION

Smallholder dairy farming contributes towards resilience to food (nutrition) insecurity, and income in rural households (Banda et al., 2021), hence the need to increase production and productivity of dairy cattle. This notwithstanding, the demand for animal protein doesn't match the supply, and is expected to increase consequent to increasing human population (Pandey, 2014; MFL, 2020; Abigaba et al., 2022). Of stark interest, however, is the observation that the full production potential of dairy cattle has not been fully realized. This warrants strategies to improve reproductive efficiency, and thus production of dairy cattle (Pandey, 2014; Hadgu and Fesseha, 2020). Artificial insemination (AI) is one crucial biotechnology used in dairy farming to improve genetic and reproductive performance (Akhtar et al., 2007). However, the lower than expected conception rates attributable to factors like inappropriate heat detection, poor insemination techniques, inter alia, observed especially in rural areas, have been reported (Akhtar et al., 2007). This poor performance inevitably attracts economic losses among farmers. Moreover, many smallholder farmers in Zambia, and elsewhere, are unable to know whether or not a cow is pregnant. Therefore, one collateral consequence of this inadequacy is the culling and disposal of an inseminated cow at a time when it could actually be pregnant (Zulu et al., 2013).

One indispensable and crucial on-farm strategy to improve performance of dairy cattle is pregnancy detection (Crowe et al., 2018). Establishing a timeous and efficient pregnancy detection routine, to accurately identify pregnant and non-pregnant cows, allows for the producers to make timely management decisions including culling of infertile females or re-synchronizing non-pregnant ones (Dilrukshi and Perera, 2009; Balhara et al., 2013; Fontes et al., 2022). When non-pregnancy is detected early, the calving interval is shortened, and the animal's life-time production is increased (Balhara et al., 2013; Aswathnarayanappa et al., 2019). With the heightened desire to utilize AI (MFL, 2020), failure to conduct timeous non-pregnancy detection is an economic drain on farm financial resources (Fontes et al., 2022). There are many pregnancy detection techniques used in cattle, notably rectal palpation, ultra-sound technique, milk progesterone test, Punyakoti (seed germination inhibition) test and other hormonal assays (Lavon et al., 2022).

Despite there being a number of techniques, most pregnancy diagnostic tools are very technical, invasive, and cost prohibitive (Dilrukshi and Perera, 2009; Skálová et al., 2017) and some require prior experience. Although rectal palpation is the most widely used of these techniques (Zulu et al., 2013; Lavon et al., 2022), Punyakoti test is far cheaper, non-invasive, and can be performed on-farm by farmers (Rine et al., 2014; Skálová et al., 2017) using readily available materials like urine and cereal seeds. This test relies on seed germination inhibition, which has been attributed to the presence or absence of hormonal metabolites and substances, in urine, such as abscisic acid and/or steroids like progesterone or estrogens (Rine et al., 2014; Okunlola et al., 2019).

There are reports of Punyakoti test application in many countries (Rahman and Saha, 2020) with some of them repeatedly recommending its application in pregnancy detection (Rine et al., 2014; Rahman and Saha, 2020). However, to date, dairy farmers in Zambia have not utilized Punyakoti test probably because, like other livestock farmers, their awareness of pregnancy detection technologies is generally low (Abigaba et al., 2022). In addition, there are currently no reports on Punyakoti test that have been conducted in Zambia. In a previous report (van Arendonk, 2011), the promotion of tailor-made biotechnology solutions which meet the needs of farmers and the demands of consumers was recommended. This creates an urgent need to validate Punyakoti test for use in dairy cows reared by smallholder farmers under tropical conditions. While several previous studies have used wheat seeds (Rine et al. 2014; Juodžentytė and Žilaitis 2016; Skálová et al. 2017; Rai et al. 2018), few studies have employed maize seeds which are the main and readily available cereals in Zambia. Furthermore, results associated with Punyakoti test obtained thus far are replete with variations, which could be attributed to differences in seed type, moisture, day length, nutrition, and temperature (Bowden and Ferguson, 2008). This study was therefore conducted to validate Punyakoti test application in dairy cattle under smallholder farming system with the objectives tailored to (1) assess the germination inhibition potential of urine from dairy cattle reared by smallholder farmers, (2) evaluate the earliest period, after insemination, during which smallholder dairy farmers can reliably apply the Punyakoti test, and (3) establish the level of association between the treatment factor and seed germination and/or shoot lengths.

MATERIALS AND METHODS

Study site and experimental animals

The study was carried out at Kanakantapa, Chongwe District of Lusaka, Zambia. The handling of animals and experimentation were done with routine supervision by the institutional committee on the animal research, and in compliance with the guide for the care and use of agricultural animals in research and teaching (FASS, 2020).

The experiment

Procedure: A total of eighteen (18) dairy animals, that included 13 dairy cows and five heifers, were randomly selected from different smallholder farms in Kanakantapa area. Out of 13 dairy cows, eight of them were randomly selected for synchronization, and then inseminated using AI. With regard to the trial set up, four treatment groups were used: eight inseminated dairy cows (AIC), positive (pregnant) group; 5 non-inseminated dairy cows (non-AIC), negative group; 5 non-inseminated heifers (non-AIH), negative group; and distilled water (DW), as the control group. To ensure quality, certified pure maize seeds used in the study were obtained from Department of Plant Science, School of Agricultural Sciences, The University of Zambia. Free-catch urine samples used were obtained from the experimental animals between 06:00 and 07:00

hours of each sample collection day; free catch method was preferred because its non-invasive, and the collection early in the morning aimed to obtain urine concentrated with metabolites and substances of study interest. The collection was done, in 200 mL sample bottles, on day 15, 22, 30, and 35 after insemination. The procedures for urine collection, its preparation, and treatment with maize seeds were based on a previous study (Juodžentytė and Žilaitis, 2016), with some modification to suit the current study objectives. Briefly, each collected urine sample was diluted using distilled water, in the ratio of 1:4, and 15 mL of the diluted urine was introduced onto 15 maize seeds placed on filter paper in a petri dish. Each treatment sample was done in triplicate. The same procedure was followed for seeds in the control group wherein only distilled water was applied in place of urine. All treatment petri dishes containing maize seeds were stored covered, at an average temperature of 25°C, and after three days, seed germination percentage and shoot length were determined.

Data Collection: For each petri dish, the number of seeds that germinated and the total number of seeds in a petri dish were used to compute the maize seed germination percentage. The germination percentage (rate) was calculated using the formula;
Germination percentage (%)= number of maize seeds that germinated/total number of seeds in petri dishes x 100

Furthermore, the shoot lengths (cm) of germinated maize seeds were measured using a ruler. The average value for germination percentage and that of shoot length, for each treatment sample, were computed.

Data analysis: The data on maize seed germination and shoot lengths were analysed descriptively, in statistical package for social scientists (SPSS® IBM 26 version, USA), to obtain the means and standard errors of means (SE). The differences between treatment group means, namely AIC, non-AIC, non-AIH, and DW on different days after insemination were analysed using Kruskal-Wallis test. Where a difference was indicated, a Bonferroni (post hoc) test was performed to identify the pair with significant differences. The association between selected parameters was analysed using the Eta coefficient test statistic. The significance level was accepted at P<0.05.

RESULTS

The means of germinated maize seeds in different treatment groups, and in each treatment group on different days after AI

The germination percentages of seeds in different treatment groups, namely inseminated dairy cows' urine (AIC), non-inseminated dairy cows' urine (non-AIC), non-inseminated heifers' urine (non-AIH), and distilled water (DW) are presented in Table 1. The mean germination percentage of seeds was lowest (21.92±1.15) in AIC group on day 35; the mean germination percentage of seeds on the same day was highest (84.00±1.95) in DW. On different days, the mean germination percentages were higher in DW than other treatment groups, with the highest mean (86.66±3.50) observed on day 22. Kruskal-Wallis test revealed significant differences in mean values of germinated seeds between treatment groups on day 30 (P<0.05) and day 35 (P<0.05), and no level of significance was observed on the day 15 (P>0.05) and day 22 (P>0.05). The Bonferroni test revealed that mean of germinated seeds in AIC group differed significantly from that in non-AIC (P<0.05), non-AIH (P<0.05), and DW (P<0.05) on day 30. Similarly, the mean values in AIC differed significantly from that in non-AIC (P<0.05), non-AIH (P<0.05) and DW (P<0.05) on day 35.

Furthermore, the results of the mean of germinated seeds in each group on different days are presented in table 1. In AIC group, the lowest mean value (21.92±1.15) was observed on day 35, while the highest (84.50±1.61) was on day 15. The lowest mean value in non-AIC was 80.32±2.41 on day 35; 80.12±1.31 in non-AIH on day 30; and 84.00±1.95 in DW on day 35. The highest mean value in non-AIC was 83.94±1.91 on day 15; 83.49±3.16 in non-AIH on day 15; and 86.66±3.50 in DW on day 22. Kruskal-Wallis test revealed that the means of germinated seeds in AIC group, on different days after insemination, differed significantly (P<0.05). The Bonferroni test showed a significant difference between the mean values at day 30 and 22 (P<0.05), day 30 and 15 (P<0.05), day 35 and 22 (P<0.05), as well as day 35 and 15 (P<0.05); the mean values didn't differ significantly for day 22 and 15 (P>0.05) as well as day 35 and 30 (P>0.05). The Kruskal-Willis test did not reveal significant differences (P>0.05) in means of the germinated seeds, between the days after insemination, in other treatments.

The mean shoot lengths of maize seeds in different treatment groups on different days after AI

Mean shoot lengths of seeds in different groups, namely AIC, non-AIC, non-AIH and DW are presented in Table 2. The mean shoot lengths (cm) was lowest (0.91±0.05) in AIC group on day 35; the mean shoot lengths of seeds on the same day was highest (3.05±0.14) in DW. On the different days, the mean shoot lengths were higher in DW than other treatment groups, of which the highest (3.06±0.24) was observed on day 22. Kruskal-Wallis test revealed significant differences in mean shoot lengths between treatment groups for day 30 (P<0.05) and day 35 (P<0.05), and no level of significance was observed on day

Table 1: Mean germination percentages of maize seeds in different groups on different days after AI

Variables	Days post-insemination			
Groups	Day 15	Day 22	Day 30	Day 35
AIC	84.50±1.61*A	82.40±0.67 A	25.52±1.18B	21.92±1.15B
Non-AIC	83.94±1.91 A	82.91±2.61 A	81.96±2.61 A	80.32±2.41 A
Non-AIH	83.49±3.16 A	80.80±2.05 A	80.12±1.31 A	80.87±1.50 A
DW	86.00±3.01 A	86.66±3.50 A	85.58±0.62 A	84.00±1.95 A

Note: *Mean±SE, Groups with dissimilar superscripts (A, B) within a column differ significantly (*P*<0.05), Groups within dissimilar superscripts (A, B) within a row differ significantly (P<0.05), AIC: Inseminated cows, Non-AIC: Non-inseminated cows, Non-AIH: Non-inseminated heifers.

Table 2: Mean shoot lengths of maize seeds in different groups on different days after AI

Variables	Days after insemination			
Groups	Day 15	Day 22	Day 30	Day 35
AIC	2.92±0.08*B	2.83±0.03B	1.02±0.05C	0.91±0.05C
Non-AIC	2.93±0.11B	2.84±0.14B	2.94±0.06B	2.46±0.47B
Non-AIH	2.83±0.14B	2.87±0.01B	2.90±0.08B	2.73±0.08B
DW	3.05±0.06B	3.06±0.24B	3.00±0.10B	3.05±0.14B

Note: *Mean±SE, Groups with dissimilar superscripts (B, C) within a column differ significantly (*P*<0.05), AIC: Inseminated cows, Non-AIC: Non-inseminated cows, Non-AIH: Non-inseminated heifers.

Table 3: The Eta coefficient values relating the treatment factor and seed germination and shoot length variables

Variable	Seed germination				Shoot length			
	Day 15	Day 22	Day 30	Day 35	Day 15	Day 22	Day 30	Day 35
Relationship factor	0.149	0.364	0.992	0.993	0.166	0.290	0.989	0.876

Note: <0.2: No association, 0.2-0.39: Weak association, 0.4-0.69: Medium association, >0.7: Strong association

15 (P>0.05) as well as day 22 (P>0.05). The Bonferroni test revealed that mean shoot lengths of seeds in AIC differed significantly from that in non-AIC (P<0.05), non-AIH (P<0.05), and DW (P<0.05) on day 30. Similarly, the mean values in AIC differed significantly from that in non-AIC (P<0.05), non-AIH (P<0.05), and DW (P<0.05) at day 35.

The relationship between the treatment factor and maize seed germination and shoot lengths on different days after AI.

The results for relationships between the treatment groups (factor) and seed germination and shoot lengths variables are presented in Table 3. In the current study, spearman's correlation analysis revealed a significant relationship (r = 0.786; P<0.05) between maize seed germination and the shoot lengths variables. The highest and lowest Eta coefficient values for the association between the treatment factor and seed germination variable were 0.993 and 0.146, respectively; the highest and lowest Eta coefficient values for treatment factor and shoot lengths association were 0.876 and 0.166, respectively.

DISCUSSION

One of the most important management activities needed to pursue, for optimal reproductive performance, is diagnosis of early pregnancy in dairy cows (Crowe et al., 2018; Szenci, 2021). Accordingly, there are many techniques that can be used to diagnose pregnancy, for example ultrasound scanning, rectal palpation, hormonal assay, and the milk progesterone test (Rahman and Saha, 2020; Fontes et al., 2022; Lavon et al., 2022). However, many farmers especially those under smallholder dairy farming systems are unable to diagnose pregnancy (Zulu et al., 2013), particularly employing these techniques. This contributes to low performance and in turn economic losses. Thus, the promotion of Punyakoti technique for use in dairy cattle pregnancy diagnosis remains crucial, more so in the rural areas of Zambia. Cognizant of its relative advantages (Dilrukshi and Perera, 2009; Rao Krishna and Veena, 2009), the current study findings will likely benefit the efforts to improve reproductive performance of dairy cattle, under smallholder farming, through routine pregnancy detection using the Punyakoti technique. To date, there are limited reports of its application in the Sub-Saharan Africa even though it originated from the ancient Egypt (Bethapudi et al., 2015). This could be attributed to a lack of awareness of this technique and any other pregnancy detection techniques as is the case with other livestock farmers (Abigaba et al., 2022).

The current higher mean germination and shoot length of maize seeds cultured in DW than that placed in urine-water solutions agreed with previous studies that used maize seeds (Okunlola et al., 2019) and other cereal seeds like wheat seeds (Rine et al., 2014; Skálová et al., 2017), and mung beans (Lázničková et al., 2020). Our findings support Lázničková et al. (2020) who reported that the mean values were higher in DW than urine-water solutions regardless of the type of seeds used. The same trend is true for this study and the previous ones that used maize seeds in Zebu cows (Okunlola et al., 2019), dairy cows (Hussain et al., 2016), and heifers (Skálová et al., 2017), confirming that the mean germination and shoot length of maize seeds in DW is higher than that in urine-water solutions regardless of the breed or parity. Per contra, for each treatment group, there were variations in the figures between the current study and those reported in earlier studies (Rao Krishna and Veena, 2009; Rine et al., 2014). These variations could be attributed to factors such as moisture content, temperature, day light, seed type and storage (Bowden and Ferguson, 2008), number of seeds per petri dish, and turnaround time considered.

Furthermore, the significantly different mean germination as well as the shoot lengths of seeds in the inseminated and non-inseminated groups on day 30 and 35 supports the earlier findings by Rine et al. (2014) and Rai et al. (2018) who attributed this difference to pregnancy. Since most inseminated cows conceive (Shanku, 2022) and were confirmed to be pregnant; the observed difference in the current study validates the potential of Punyakoti test to detect pregnancy, after AI, in dairy cattle under smallholder farming. Rine et al. (2014) suggested that the difference in germination percentage as well as the shoot length of seeds in different treatment groups could be attributed to hormones, hormonal metabolites and/or substances which are present in urine.

With regard to hormonal metabolites and other chemical substances found in urine, many studies have pinpointed abscisic acid, auxins, and steroids like progesterone and 17β-estradiol as the main cause of the inhibition of seed germination and shoot growth (Veena et al., 2003; Rine et al., 2014; Lázničková et al., 2020). It was also confirmed (Veena et al., 2003; Rine et al., 2014) that their levels are significantly higher in urine from pregnant cows than non-pregnant ones. For example, the levels of steroids such as progesterone, which is confirmed to inhibit germination and shoot growth, are higher in pregnant cows (Lázničková et al., 2020) because of their increased secretion from the corpus luteum and/or placenta. Additionally, the levels of abscisic acid are higher in urine from pregnant cows (170.62 nanomoles/mL of urine) than in non-pregnant cow urine (74.46 nanomoles/mL) (Veena et al., 2003). Rao Krishna and Veena, (2009) suggested that plant growth promoters like abscisic acid are likely to be excreted in urine as and when animals feed on plants containing such substances. Whether higher levels in pregnant animals follow physiological processes intended to excrete abscisic acid perhaps as a strategy to prevent possible antagonistic effects with reproductive hormones or arrest of embryonic/foetal growth and development; or excreted because of a mere embryo/foetal interaction with the dam, warrant further research. In plants, however, its presence inhibits or lowers germination rate and growth through a reversible embryo arrest, osmotic regulation with water uptake inhibition, and bud dormancy (Tuan et al., 2018; Chen et al., 2019).

Although Punyakoti technique is cheap, non-invasive and generally simple to perform (Rine et al., 2014; Skálová et al., 2017), farmers will likely favour additional attributes such as efficacy for true pregnancy or non-pregnancy status and rapidity of the test. Thus, the turnaround time of five days, with this technique, and the uncertainty over the earliest time to conduct the test with desirable efficacy are crucial factors to deal with (Rao Krishna and Veena, 2009; Rahman and Saha, 2020). Towards sound decision-making in regard to the day, after insemination, which is suitable for farmers to conduct Punyakoti test, this study revealed significant differences in mean germination values between AIC and non-AIC and AIH on day 30, and not on day 22. Furthermore, there was gradual increase in the mean values from day 15 to day 22 and a significant increase from day 22 to day 35 which agreed with earlier reports in in India and Bangladesh (Rao Krishna and Veena, 2009; Rine et al., 2014). The current findings suggest that Punyakoti technique can be used to detect pregnancy in dairy cattle under smallholder farming conditions, with acceptable results, if it is conducted on the day towards month-end after insemination. This supports some earlier studies which reported day 28 with an efficacy of over 60% (Rao Krishna and Veena, 2009; Hussain et al., 2016) and days 28 to 35 with almost 100% efficacy (Rine et al., 2014).

It is confirmed that Punyakoti technique is generally simple, and relies on the percentage germination inhibition and shoot length of the seeds (Hussain et al., 2016; Skálová et al., 2017). However, not many ordinary smallholder dairy farmers can perform shoot length measurements with accuracy, hence the counting of seeds that germinated or did not germinate to detect pregnancy status is a more feasible option. Accordingly, this study explored the association between treatment groups (factor) and seed germination and shoot length variables. Understanding the dependent variable with a higher correlation value would assist in guiding the farmers on a more reliable option to use. In the current study, Spearman's correlation analysis confirmed that both seed germination and shoot length variables are strongly correlated, with either of which being influenced by the independent factor (treatment factor). Thus, a farmer could rely on one of dependent variables to detect pregnancy; however, choice of the variable largely depends on the level of its association with the independent factor. The Eta analysis revealed that treatment factor is more associated with seed germination than it is with shoot length variable. Therefore, it is possibly meaningful, considering the time and simplicity factors, for dairy farmers to rely on seed germination parameter to detect pregnancy.

CONCLUSION

Smallholder dairy farming contributes to farmers' resilience to food (nutrition) insecurity, and income in rural households. Thus, increasing the reproductive performance and production of dairy cattle through early pregnancy detection is crucial. The current study findings confirm that Punyakoti technique can be applied by farmers to detect pregnancy in dairy cattle under smallholder farming. When maize seeds are used, a desirable efficacy for true pregnancy detection is expected if the test is conducted on the day somewhere towards month-end after AI. The treatment factor is more associated with seed germination than shoot length variable; thus, farmers may rely on seed germination parameter to detect pregnancy in dairy cattle with acceptable results.

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CONFLICT OF INTEREST

Authors declare no conflict of interest regarding this publication.

NOVELTY STATEMENT

This study investigated the utility of Punyakoti test technique for pregnancy detection in dairy cattle reared under smallholder farms, and confirmed that the technique can be applied with acceptable efficacy for true pregnancy when performed, using maize seeds, towards month-end after AI. For the first time, the study has confirmed that seed germination variable is more correlated with the treatment factor, and may be relied on to detect pregnancy with more desireable results, compared to shoot length variable.

AUTHORS' CONTRIBUTION

Pharaoh C. Sianangama conceived, designed, and supervised the study, Brian Tembo designed study and collected the data, Rubaijaniza Abigaba analysed data and wrote the manuscript, Sylivia J. Harrison supervised study and reviewed the manuscript. All authors read and approved the final manuscript for publication.

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