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Accuracy of ultrasonography and pregnancy-associated glycoprotein test for pregnancy diagnosis in buffaloes

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Abstract

The aims of the present study were to evaluate and compare the accuracy of transrectal ultrasonography and pregnancy-associated glycoprotein radioimmunoassay (PAG-RIA) test for diagnosis of pregnancy in buffaloes.

Two hundred and seventy-five buffalo cows and heifers were examined once for pregnancy diagnosis by transrectal ultrasonography using a 5 MHz linear-array transducer between Days 19 and 55 after mating. After ultrasound scanning, a blood sample was withdrawn from jugular vein of each animal for measuring pregnancy-associated glycoprotein using a heterologous double-antibody RIA. Based on palpation of the uterus per rectum at Days 75–90, 87 animals were designated pregnant and 188 as non-pregnant. The sensitivity of transrectal ultrasonography at Days 19–24 was 44.4%, reaching 100% from Day 31 after mating. The specificity of transrectal ultrasonography ranged between 92.5 and 100% from Days 19 to 55 after mating. The sensitivity of PAG-RIA test was 11.1% at Days 19–24 and reached 100% from Day 31 after mating. The specificity of PAG-RIA test ranged from 90 to 100% from Days 19 to 55 after mating. There were no significant differences between the sensitivity and specificity of the two tests in all examined periods. In conclusion, transrectal ultrasonography and PAG-RIA test are highly accurate tests for detecting pregnant buffaloes from Day 31 after mating onwards.

Keywords: Pregnancy diagnosis; PAG; Transrectal ultrasonography; Buffalo

1. Introduction

Prolonged calving intervals and poor estrus expression are major limiting factors for improving reproductive performance of buffalo cows [1]. Early and accurate pregnancy diagnosis is a key factor to shorten the calving interval. It enables the producer to identify open cows and then treat and/or rebreed them in due time.

Several methods have been used for pregnancy diagnosis in buffalo cows. Non-return to estrus is not an accurate method for early pregnancy diagnosis due to poor estrus expression in buffaloes [2]. Measurement of progesterone (P4) in blood or milk by radioimmunoassay (RIA) or enzyme immunoassay (ELISA) at Days 20–24 after breeding has been used for selecting non-pregnant buffalo cows with an accuracy reaching 100%. However, the accuracy of the test for selecting pregnant buffalo cows was low ranging from 57.1 to 75% [3–7].

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Palpation per rectum is a simple and economic method for pregnancy diagnosis; however, this method is only accurate from Days 45 of pregnancy in buffaloes [8]. In addition, palpation of fluctuation within the uterus, identification of the amniotic vesicle and slipping of the chorioallantoic membranes may increase the incidence of fetal mortality [9].

By using a 5 MHz linear-array transrectal transducer, the embryonic vesicle and embryo proper were first observed in all pregnant buffalo cows between Days 19 and 22 after AI [2]. In an experimental study, Egyptian buffalo cows ($n = 12$) were repeatedly examined by means of transrectal ultrasonography (5 MHz) [10]. In this investigation, the accuracy for selecting pregnant and non-pregnant cows at Day 21 post-AI were 89 and 75%, respectively, increasing to 100% at Days 30–35 [10]. According to Glatzel et al. [11] accurate pregnancy diagnosis with ultrasonography is only possible in buffalo heifers at Day 28 after service. In a field study using 260 buffaloes between Days 30 and 45 of gestation, sensitivity of detection of pregnancy was 97.9% when confirmation took place by manual palpation at 3 months of pregnancy [12].

Pregnancy-associated glycoproteins (PAG), also called pregnancy specific protein B (PSPB) are members of the aspartic proteinase gene family [13]. They are synthesized by binucleate trophectoderm cells (BNC), which originate from the mononucleate chorionic epithelium [14]. The BNC invade the endometrial epithelium and secrete PAG continuously throughout gestation [15,16]. Therefore, these glycoproteins are good indicators of a live conceptus [17]. Several PAG molecules have been isolated and purified. Among these glycoproteins, bovine (bo) PAG 67 kDa [18] and caprine PAG 55, 59 and 62 kDa [19] are closely related to each other.

Radioimmunoassay has been developed for the measurements of PSPB/PAG concentrations and used for accurate pregnancy diagnosis in cattle [20,21], sheep [22], goats [23] and deer [24]. The RIA test for bovine PSPB was able to detect the analogous antigen in buffalo cows from Day 30 after breeding. On Day 35 after breeding, 91% of pregnant buffalo cows had PSPB blood level higher than 1 ng/mL [25]. Unlike cattle, blood levels of PSPB or PAG in pregnant buffalo cows are low and constant throughout pregnancy and do not greatly increase before calving [26]. After calving, the blood levels of PSPB are detectable up to Day 50 postpartum [25]. Debenedetti et al. [27] compared three RIA tests using different antisera raised against $boPAG_{67\ kDa}$, $caPAG_{55+59\ kDa}$ and $caPAG_{55+62\ kDa}$ for detecting the PAG molecules in buffaloes and they found that buffalo PAG are better recognized by antisera raised against caprine PAG. To the best of our knowledge, the accuracy of PSPB or PAG-RIA test for diagnosing early pregnancy in buffaloes has not been evaluated.

The aims of the present study were to evaluate and compare the accuracy of transrectal ultrasonography and PAG-RIA tests for diagnosis of pregnancy in buffaloes.

2. Materials and methods

2.1. Animals and their breeding

This study was carried out on 275 Egyptian buffalo cows and heifers (*Bubalus bubalis*) during the period from August 2004 to March 2005. The animals were kept in Mehallet Mousa Research Station belonging to Animal Production Research Institute, Agriculture Research Center. The animals were observed for estrus twice a day and those showing signs of estrus were mated by fertile bulls. Date of mating was considered as Day 0 for calculating the gestational age.

2.2. Ultrasonographical examinations

The period between mating and pregnancy testing ranged from Days 19 to 55. Transrectal ultrasonography and PAG-RIA were applied once in all animals from Days 19 to 55. A real time B-mode ultrasound scanner (Ultrascan 900, Alliance Inc., Amos Lachine, Quebec, Canada) equipped with a 5 MHz linear-array transducer was used for transrectal ultrasonographic examinations. All animals were examined by the same operator who had experience for pregnancy diagnosis by ultrasonography. The animals were restraint in stanchion with dim ambient lighting. The operator introduced his lubricated gloved hand into the rectum. After removal of feces, the transducer was inserted into the rectum and was slowly moved from the cervix to the right and then to the left uterine horn until the entire uterus was imaged. The ovaries were not involved in the scanning. Recognition of anechoic (allantoic) fluid and/or the embryo proper with a beating heart was used as a positive sign for pregnancy [21].

2.3. Blood sampling and PAG-radioimmunoassay

After ultrasound scanning, a blood sample was withdrawn from the jugular vein of each animal. Immediately after collection, blood samples were centrifuged at $1500 \times g$ for 20 min. The collected serum was stored at $-20\ ^{\circ}C$ until analysis of PAG.

Concentrations of serum PAG were initially measured in a pre-incubated PAG-RIA test, as previously described and validated by Perenyi et al. [28,29]. Bovine $PAG_{67\ kDa}$ was used as tracer and standard. Rabbit antiserum raised against a mixture of $caPAG_{55+62\ kDa}$ (R#706) was used as the first antibody at an initial dilution of 1:80,000.

Samples with PAG concentrations beyond the range of the standard curve of the assay were re-assayed in a non-preincubated system. In this system, antiserum R#706 was used at an initial dilution of 1:120,000. Minimum detection limits were 0.10 and 1.60 ng/mL for pre-incubated and non-preincubated systems, respectively. The intra-assay coefficients of variation ranged from 3.7 to 5.5%.

The cut-off value of PAG-RIA test used to detect pregnant buffalo cow was $\geq$1.8 ng/mL. At this cut-off value, there was the least difference between the sensitivity and specificity of the test for pregnancy diagnosis. A similar approach to find a cut-off value has been used before in sheep [30].

2.4. Analysis of data

Pregnancy diagnoses were confirmed by palpation of the uterus per rectum at Days 75 to 90 after mating, calving records and/or by observed event such as abortion. The results of transrectal ultrasonography and PAG-RIA were arranged as follows: correct positive diagnosis (a), incorrect positive diagnosis (b), correct negative diagnosis (c), and incorrect negative diagnosis (d). From these values the sensitivity ($100 \times a/a + d$), the specificity ($100 \times c/c + b$), the positive predictive value ($100 \times a/a + b$) and the negative predictive value ($100 \times c/c + d$) of each test were calculated [31]. A Chi-square test was used to compare the sensitivities and specificities of the two pregnancy tests [32]. Differences between pregnant and non-pregnant buffaloes in the level of PAG were statistically analyzed by using a Student's *t*-test [33].

3. Results

Based on palpation of the uterus per rectum at Days 75–90, 87 animals were designated pregnant and 188 as non-pregnant. The accuracy of transrectal ultrasonography for pregnancy diagnosis in buffalo cows is shown in Table 1. The sensitivity of the test was low at Days 19–24, increased with progression of pregnancy to reach 100% from Days 31 to 35 of gestation (Table 1). Altogether seven false-negative diagnoses were made by means of transrectal ultrasonography at Days 19–55 after mating. The specificity of the test was relatively high and steady between Days 19 and 35 and reached 100% from Days 36 to 40 of gestation. Eight false-positive diagnoses were made by means of transrectal ultrasonography; three of them had PAG concentration higher than the threshold of diagnosing pregnancy (Table 3).

The accuracy of PAG-RIA test for pregnancy diagnosis in buffalo cows is shown in Table 2. The sensitivity of the test was very low (11.1%) at Days 19–24, increased to 80% at Days 25–30 and reached 100% at Days 31–35 of gestation (Table 2). Eleven false-negative diagnoses were made by PAG-RIA test at Days 19–55 after mating. The specificity of the test was high and constant (>90%) from Days 19 to 55 after service. Altogether 12 false-positive diagnoses were made by the test during all the examined periods; three of them were also positive by the ultrasound test (Table 3) and the remaining ones were negative.

There were no significant differences between the sensitivity and specificity of transrectal ultrasonography and PAG-RIA test during all examining periods.

The levels of PAG in pregnant and non-pregnant buffaloes as determined by rectal palpation between

Table 1
Sensitivity (Se), specificity (Sp) and predictive values (+PV; −PV) of a 5 MHz transrectal ultrasonography for early pregnancy diagnosis in buffaloes ($n = 275$)

Grouping and evaluation	Days 19–24 ($n = 62$)	Days 25–30 ($n = 45$)	Days 31–35 ($n = 39$)	Days 36–40 ($n = 35$)	Days 41–45 ($n = 20$)	Days 46–50 ($n = 31$)	Days 51–55 ($n = 43$)
a	4	13	8	12	9	16	18
b	4	2	2	0	0	0	0
c	49	28	29	23	11	15	25
d	5	2	0	0	0	0	0
Se (%)	44.4	86.7	100	100	100	100	100
Sp (%)	92.5	93.3	93.5	100	100	100	100
+PV (%)	50	86.7	80	100	100	100	100
−PV (%)	90.7	93.3	100	100	100	100	100

a, correct positive diagnosis (pregnant); b, incorrect positive diagnosis (non-pregnant); c, correct negative diagnosis (non-pregnant); d, incorrect negative diagnosis (pregnant).

Table 2
Sensitivity (Se), specificity (Sp) and predictive values (+PV; −PV) of pregnancy-associated glycoprotein RIA test for early pregnancy diagnosis in buffaloes

Grouping and evaluation	Days 19–24 ($n = 62$)	Days 25–30 ($n = 45$)	Days 31–35 ($n = 39$)	Days 36–40 ($n = 35$)	Days 41–45 ($n = 20$)	Days 46–50 ($n = 31$)	Days 51–55 ($n = 43$)
a	1	12	8	12	9	16	18
b	2	3	3	2	0	1	1
c	51	27	28	21	11	14	24
d	8	3	0	0	0	0	0
Se (%)	11.1	80.0	100	100	100	100	100
Sp (%)	96.2	90	90.3	91.3	100	93.3	96.0
+PV (%)	33.3	80	72.7	85.7	100	94.1	94.7
−PV (%)	86.4	90.0	100	100	100	100	100

a, correct positive diagnosis (pregnant); b, incorrect positive diagnosis (non-pregnant); c, correct negative diagnosis (non-pregnant); d, incorrect negative diagnosis (pregnant).

Table 3
Concentrations (ng/mL) of pregnancy-associated glycoprotein (PAG) and ultrasound (US) findings in three cows (palpated as non-pregnant between days 75 and 90) with presumed embryonic mortalities

Cow	Days of pregnancy	US	PAG (ng/mL)
A	24	P	1.88
B	27	P	2.17
C	33	P	2.27

P, pregnant.

days 75 and 90 are shown in Table 4. The level of PAG in pregnant buffaloes was significantly higher ($P < 0.05$, $P < 0.001$ and $P < 0.0001$) than that in non-pregnant cows in all periods of examination.

4. Discussion

The sensitivity of transrectal ultrasonography at Days 19 to 24 (44.4%) was much lower than that (100%) reported by Pawshe et al. [2] who detected the embryo proper in all examined buffalo cows ($n = 26$) by using a 5 MHz linear-array transducer at Day 19. Also it was lower than that (88.9%) reported by El-Shahat et al. [10] at Day 21. Unlike the present field study, examination of the animals by means of ultrasonography in the other two studies was carried out under experimental conditions, whereas the same animal was scanned at repeated intervals. Similar low sensitivity of transrectal ultrasonography was reported in a field study carried out in dairy cattle at Days 21–25 of gestation [34]. A small amount of allantoic fluid appeared difficult to be detected between Days 19 and 24 of gestation resulting in higher frequency of false-negative diagnoses. In the present study, the sensitivity of the test increased from Day 25 and reached 100% from Days 31 to 35 of gestation. These findings could be attributed to an increasing amount of allantoic fluid within the uterine horn and the detection of embryo proper in most of pregnant animals. The same sensitivity level was reported by El-Shahat et al. [10] at Days 30–35 of gestation.

The specificity of transrectal ultrasonograhy at Days 19–24 (92.5%) was higher than that (75%) reported at Day 21 of gestation in one study [10], in which only a very small number of non-pregnant ($n = 3$) animals were used. The increased uterine fluid around the period of estrus and the occurrence of early embryonic mortality might have contributed to the false-positive

Table 4
Concentrations (mean ± S.D.) of pregnancy-associated glycoprotein (PAG) (ng/mL) in pregnant and non-pregnant buffaloes

Animals	Days after mating						
	1–24	25–30	31–35	36–40	41–45	46–50	51–55
Pregnant	1.6 ± 1.2^{a}	6.4 ± 4.6^{c}	9.4 ± 4.5^{a}	10.9 ± 6.7^{c}	24.4 ± 5.9^{e}	35.0 ± 21.7^{e}	40.6 ± 19.3^{e}
n	9	15	8	12	9	16	18
Non-pregnant*	0.6 ± 0.5^{b}	0.8 ± 0.6^{d}	1.0 ± 0.6^{b}	1.1 ± 1.4^{d}	0.5 ± 0.4^{f}	0.6 ± 0.6^{f}	0.6 ± 0.6^{f}
n	53	30	31	23	11	15	25

*Animals with false-positive diagnoses were also involved in the calculation.
[a,b] $P < 0.05$.
[c,d] $P < 0.001$.
[e,f] $P < 0.0001$.

diagnoses. These explanations are supported by the findings of the present study, whereas four out of eight false-positive diagnoses made by transrectal ultrasonography were at Days 19–24 after service when estrus can be expected to occur in non-pregnant animals. Furthermore three of eight buffaloes with false-positive diagnoses (1 at Day 24, 1 at Day 27 and 1 at Day 33) had PAG level higher than the threshold (1.8 ng/mL) for pregnancy indicating that these animals were pregnant or had been pregnant but experienced embryonic mortality.

The sensitivity of PAG-RIA test was very low (11.1%) at Days 19–24, increased to 80% at Days 25–30 and reached 100% at Days 31–35 of gestation. Similar findings were reported in one study [25], in which PSPB was detectable in 33% of pregnant buffaloes at Days 20–25 of gestation, while 91% of pregnant buffaloes had PSPB level higher than the threshold for diagnosing pregnancy at Day 35 of gestation. Since pregnancy proteins are synthesized by binucleate cells, originating from the trophoblast, which start to migrate into the endometrium around the time of final attachment to the endometrium, the level of PAG was low at Days 19–24 of gestation, resulting in higher frequency of false-negative diagnoses (8/9).

Twelve false-positive diagnoses were made by PAG-RIA test. Three of them were diagnosed pregnant with US indicating that these animals were pregnant and embryonic mortalities might have occurred after examination. The remaining nine cows were ultrasound negative; these cows might have experienced embryonic mortality before scanning. Since PSPB has a relatively long half-life after calving in buffalo-cow ($\sim$10 days) [25] and PAG has about a 3-day half-life after experimentally induced embryonic mortality in heifers [35], residual quantities of the proteins may have persisted in the maternal circulation till the time of sampling.

There were no significant differences between transrectal ultrasonography and PAG-RIA test for detecting pregnant and non-pregnant buffaloes between Days 19 and 55 after mating. Transrectal ultrasonography has the advantage over PAG-RIA of being an on-farm test. Currently efforts are being made to develop accurate ELISA kits for PAG to enable the buffalo's producer to apply the test on farm, thus overcoming the lab expenses, the radioactivity hazard of using RIA method and delays in obtaining the test results. In conclusion, transrectal ultrasonography and PAG-RIA test are highly accurate tests for detecting pregnant buffaloes from Day 31 after mating onwards.

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