Research Article

Use of Bayesian Inference to Correlate *In Vitro* Embryo Production and *In Vivo* Fertility in Zebu Bulls

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The objective of this experiment was to test *in vitro* embryo production (IVP) as a tool to estimate fertility performance in zebu bulls using Bayesian inference statistics. Oocytes were matured and fertilized *in vitro* using sperm cells from three different Zebu bulls (V, T, and G). The three bulls presented similar results with regard to pronuclear formation and blastocyst formation rates. However, the cleavage rates were different between bulls. The estimated conception rates based on combined data of cleavage and blastocyst formation were very similar to the true conception rates observed for the same bulls after a fixed-time artificial insemination program. Moreover, even when we used cleavage rate data only or blastocyst formation data only, the estimated conception rates were still close to the true conception rates. We conclude that Bayesian inference is an effective statistical procedure to estimate *in vivo* bull fertility using data from IVP.

1. Introduction

The success of artificial insemination (AI) programs in cattle depends on the use of bulls with optimal fertility. Using animals with high fertility rates, maximum conception rates can be achieved during the breeding season, reducing the cost of the program. However, until now, the most efficient way to estimate the fertility of a particular bull is to use a field fertility test [1], which is very expensive and time consuming [2].

In the past decades, many studies have been performed with the objective of developing a laboratory test to evaluate semen from different animals and predict its performance after insemination. This kind of test would be beneficial, since it would reduce the probability of using low-fertility

bulls in AI programs [3] without the necessity of inseminating a large number of females to perform the bull fertility test.

Several semen characteristics have been analyzed to ensure quality and fertility. The most studied characteristics are sperm motility [4, 5] and morphology [6] and plasmatic [7] and acrosomal [8] membrane integrity. Although those characteristics have proven to be important in semen analysis, their correlation with bull fertility is very low [9], with substantial variation among studies.

In bovine IVP, the *in vitro* fertilization and the *in vitro* culture of embryos have been proposed as suitable biotechnical tools for the prediction of fertility performance in bulls [2, 10–12]. However, even though the use of IVP has led to interesting results, especially when combined

with semen analysis (motility, morphology and membrane integrity), previous studies have found conflicting results when IVP and fertility results from the same bull are compared [13, 14]. Until now, no laboratory test has successfully predicted, with good repeatability, male fertility in domestic animals.

The objective of the present study was to evaluate the application of a statistical model using Bayesian inference [15] to estimate fertility performance in Zebu bulls using data from an IVP program and the true conception rates previously obtained from each bull.

2. Materials and Methods

2.1. In Vitro Maturation. Ovaries obtained from a commercial slaughterhouse were utilized in this experiment. Oocytes were aspirated with an 18-gauge needle connected to a 10 mL syringe from follicles of 2 to 8 mm in diameter. Oocytes were selected according to cytoplasm morphology and number of cell layers in the cumulus oocyte complexes. Only oocytes surrounded by more than three layers of cumulus cells and having homogeneous cytoplasm were used. The selected oocytes ($N = 997$) were matured for 22 to 24 hours at $38.5°C$ in an incubator with 5% CO_2 in the air and 100% humidity. Oocytes (20–30 per drop) were matured in $90 \mu L$ drops covered with mineral oil. The maturation medium was composed of TCM-199 with Earle's salts and L-glutamine (Gibco 31.100, Grand Island, NY, USA) supplemented with 5 mg/mL BSA, 2.2 mg/mL sodium pyruvate, 1 mg/mL estradiol 17β, $50 \mu g/mL$ hCG (Profasi, SE, Brazil, 5.000 UI), $1 \mu g/mL$ FSH (Foltropin-V, Vetrepharm, ON, Canada), and $75 \mu g/mL$ gentamicin. All drugs were purchased from Sigma (Sigma-Aldrich Corp., St. Louis, Mo, USA) unless otherwise specified.

2.2. In Vitro Fertilization. For fertilization, commercial frozen-thawed semen of an unique batch from three different Zebu bulls were used, and all of them were Nellore breed bulls (*Bos taurus indicus*), named V, T, and G. Sperm cells from all bulls were selected through a Percoll gradient, and the concentration was adjusted to 1×10^6 sperm cells/mL. Fertilization was performed in HTF medium (Irvine Scientific, Santa Ana, Calif, USA) supplemented with 5 mg/mL BSA, 0.5 mg/mL caffeine, 2.2 mg/mL sodium pyruvate, $30 \mu g/mL$ heparin, $18 \mu M$ penicilamine, $10 \mu M$ hypotaurine, $1.8 \mu M$ epinephrine, and $75 \mu g/mL$ gentamicin. Groups of 20–30 oocytes were incubated with the sperm cells for approximately 18 hours under the same conditions described for maturation.

2.3. Evaluation of Pronuclear Formation. For pronuclear formation analysis, forty presumptive zygotes per group were denuded and stained with 1% acetic orcein (COA) for pronuclear visualization. The rate of fertilized zygotes was obtained based on the total number of presumptive zygotes in each sample. The zygotes were transferred to small drops of DPBS with 1% BSA on a histological slide and covered with a cover slip. The slides were then fixed in Carnoy's fixative. After 24 hours of fixation, the zygotes were stained with 1% acetic orcein and examined under a light microscope.

2.4. In Vitro Culture. The remaining zygotes were denuded and transferred to culture dishes containing SOFaa medium (Nutricell, Campinas, SP, Brazil). Embryos were cultured for seven days in a mixed-air incubator (90% N_2, 5% CO_2, and 5% O_2). Cleavage and blastocyst formation data were collected on days three and seven of culture, respectively, based on the total number of oocytes used in each group (except for the ones removed for COA staining).

2.5. In Vivo Bull Fertility, Cows and Artificial Insemination. The *in vivo* bull fertility was obtained after a fixed-time artificial insemination (FTAI) program with the same commercial semen batches from Nellore breed bulls (*Bos taurus indicus*) named V, T, and G used in IVF. Nonlactating mature Nellore (*Bos taurus indicus*) cows ($N = 492$; 7 to 10 years old; 441 ± 19 kg body weight) were used in this study. Cows were managed under an extensive grazing system based on tropical pastures. Free access to mineral supplement and water was allowed. Cows, at random stage of the estrous cycle (Day 0), received a Norgestomet implant in the auricular subcutaneous tissue containing 3 mg Norgestomet (Crestar, Intervet, SP, Brazil) along with 5 mg estradiol valerate and 3 mg Norgestomet i.m. (Intervet, SP, Brazil). On Day 9, the cows received a dose of 300 IU eCG i.m. (Folligon, Intervet, SP, Brazil), and the Norgestomet implant was withdrawn. On Day 11, 54 hours after Norgestomet implant withdrawn, all cows were FTAI. All FTAI were performed by only one technician, using commercial frozen-thawed semen from bulls V ($N = 149$), T ($N = 109$), and G ($N = 234$). Pregnancy diagnosis after the FTAI program, defined as true conception rate, was performed by transrectal ultrasonography (Aloka 500 V equipped with a 5.0-MHz linear array transducer) 60 days after the end of the synchronized period.

2.6. Statistical Analysis. Cleavage and blastocyst percentage data were analyzed by ANOVA followed by Tukey's test. The untransformed data is presented in Table 1. The conception rates from the three bulls were compared using a Chi-square test. As the environments and animal categories were the same, there was not any possible other significant effect. Both sets of data were analyzed using the statistical software GraphPad InStat 3.0 ($P < .05$).

In order to determine the correlation between laboratory results and the true fertility of each bull, cleavage, and blastocyst formation means were analyzed in comparison with the true conception rates for bulls V, T, and G using Bayesian inference [16]. First, a binomial model was adopted to establish linked functions and predictive models (Appendix (A), (B), and (C), resp.). Using the Bayesian procedure implemented in the program Winbugs 1.4 [15], the predictive model parameters α, β_1, and β_2 were calculated through Marcov Chain Monte Carlo (MCMC) computer algorithms using the Gibbs sampler method to establish the predictive model (Appendix (C)) based on the data of cleavage, blastocyst formation and the true conception

TABLE 1: Fertility performance of each bull expressed as conception rate after fixed-time artificial insemination (FTAI). Pronuclei visualization, cleavage rates, and blastocyst formation rates (means ± SD) observed at 12, 72, and 168 hours after in vitro fertilization, respectively.

Bull	Inseminated cows	Conception rate after FTAI (%)*	Total oocytes	Analyzed zygotes	Pronuclei (%)	Cleavage** (%)	Blastocyst** (%)
V	149	54.4	344	40	52.5 ± 1.7	77.9 ± 1.0^{b}	27.9 ± 0.5
T	109	54.1	341	40	40.0 ± 1.6	57.4 ± 0.4^{a}	22.8 ± 0.4
G	234	63.3	312	30	40.0 ± 1.8	78.1 ± 1.8^{b}	30.5 ± 0.5

[a,b]Values with different superscripts in the same column are significantly different ($P < .05$).
*Percentage calculated based on the number of pregnant cows in relation to the total number of inseminated cows.
**Percentage calculated based on the total number of oocytes minus the zygotes removed for pronuclei evaluation.

rates of bulls provided to the program. Noninformative or vague prior distributions with normal curve centered at the origin (zero) and relatively large variance were used [17]. With the predictive model established, the mean values of cleavage and blastocyst formation rates were provided to the program Winbugs 1.4 to estimate bull fertility on the basis of combined data of cleavage and blastocyst formation rates; cleavage rates alone and blastocyst formation rates alone of each bull (Appendix (C): model 1, 2 and 3 resp.).

In Appendix, first a binomial model was adopted (Appendix (A): Y_i: number of pregnant cows; N_i: total number of cows; P_i: conception rate; i: bull) to establish linked functions (Appendix (B): α, β_1 and β_2 were constants calculated in program Winbugs 1.4 through MCMC computer algorithms to establish the predictive model; Cleavage data (f_{1i}) and Blastocyst formation data (f_{2i}) that were provided to the program Winbugs 1.4 to estimate conception rates (p_i)) and finally the predictive models obtained using the program Winbugs 1.4 (Appendix (C): model 1: estimation of bull fertility based on combined data of cleavage and blastocyst formation; model 2: estimation of bull fertility based on cleavage rates only; model 3: estimation of bull fertility based on blastocyst formation rates only; f_{1i}: cleavage rate provided; f_{2i}: blastocyst formation rate provided; p_{io}: estimated bull fertility).

3. Results

Statistically significant differences were not observed among bulls in the visualization of two or more pronuclei and in blastocyst formation rates (Table 1). However, the cleavage rate observed for bull T was lower ($P < .05$) than bulls V and G (Table 1). The in vivo bull fertility data is presented in Table 1 as true conception rates after an FTAI program. All three bulls presented statistically similar fertility performance ($P = .1299$).

In this experiment, models of binomial regressions were first adopted to establish linked functions. Subsequently, the parameters α, β_1 and β_2 of the predictive models were calculated using the program Winbugs 1.4 through MCMC. Bull fertility was estimated in the program Winbugs 1.4 using provided mean data of combined cleavage and blastocyst formation rates, cleavage rates alone, and blastocyst formation rates alone (Appendix (C): model 1, 2 and 3, resp.).

Estimated conception rates and in vivo bull fertility, expressed as true conception rates, for each bull are presented in Table 2. The results show that when data from cleavage or blastocyst formation rates were used alone, the estimated conception rates were similar to true conception rate. However, when both parameters (cleavage and blastocyst formation rates) were used in combination, the estimated conception rates were nearly identical to those observed for all three bulls.

4. Discussion

The results of this experiment show that it is possible to estimate the fertility of bulls based on data obtained during IVP, using a Bayesian statistical inference model. Moreover, while the use of different bulls for in vitro embryo production has an influence on the cleavage rates of oocytes, the development of embryos until blastocyst stage becomes similar between the bulls studied.

The use of semen from different bulls influenced in vitro embryo production, since cleavage rates were different among the tested bulls (Table 1). In the same way, the use of sperm cells from different bulls during IVF results in variable fertility rates [18]. This effect, which is related to each individual male, results in variable cleavage and blastocyst formation rates and embryo viability [19, 20]. Our results are similar, since the observed cleavage rate is statistically different for bull T in comparison with bulls V and G.

Nevertheless, the percentages of pronuclear formation and blastocyst production do not differ among bulls. These results agree with the work of Shamsuddin and Larsson [21], who have demonstrated that the use of different bulls during IVF leads to different embryo developmental rates until the fourth cellular cycle (16 cells). However, when this particular developmental stage, which corresponds to embryonic genome activation, is bypassed, embryo development is similar among bulls until the morulae/blastocyst stage.

It is commonly recognized that the best method to analyze field fertility in a bull is to estimate the conception rate after an AI or natural breeding program [1]. The inconvenience of this procedure is related to the high cost and long time period necessary to obtain results [2]. Therefore, the development of an alternative laboratory test capable of

TABLE 2: Comparison between conception rate after fixed-time artificial insemination (FTAI) and estimated fertility for each bull, established using the program Winbugs 1.4 with 95% of credibility interval, based on data from cleavage, blastocyst formation rates, and on the combined data.

Bulls	Conception rate after FTAI (%)	Estimated conception rate (%±SD) and Credibility interval (%)		
		Cleavage**	Blastocyst formation***	Combined data*
V	54.4	59.8 ± 2.5 (54.9–64.5)	58.5 ± 2.2 (54.1–62.7)	54.7 ± 3.9 (47.1–62.3)
T	54.1	54.2 ± 4.7 (44.8–63.6)	52.3 ± 4.5 (43.6–61.2)	54.2 ± 4.8 (44.8–63.5)
G	63.3	59.8 ± 2.5 (54.9–64.6)	61.5 ± 2.8 (55.9–66.9)	63.0 ± 3.1 (56.9–68.9)

*Model 1 is based on combined data cleavage and blastocyst formation.
**Model 2 is based on cleavage data only.
***Model 3 is based on blastocyst formation data only (Appendix (C)). The conception rate was calculated based on the number of pregnant cows in relation to the total number of inseminated cows. Values in the same column are not significantly different ($P > .05$).

estimating the fertility of bulls would be very beneficial for the cattle industry.

Previous studies have not [13, 14] or poorly shown [22, 23] a correlation between IVP data and *in vivo* bull fertility. However, many authors indicated the benefit of using IVP data to estimate *in vivo* bull fertility [10, 11, 21, 24]. Advances in bovine IVP embryos systems have allowed the relationship between *in vivo* bull fertility and IVP outcomes to be examined [12, 25–27]. However, arising from variations in protocols between laboratories it is still unclear whether the ability of a bull to fertilize oocytes *in vitro* is useful as a predictor of *in vivo* fertility following artificial insemination [26]. In addition, it has been shown that individual bulls have marked variability in their response to *in vitro* capacitation methods [10, 28].

Zhang et al. [11] showed that both cleavage and blastocyst production rates may be positively correlated with fertility in bulls. The authors were able to determine predicted conception rates for the bulls they studied. Our experiment has produced similar findings, since the conception rates of bulls were efficiently estimated using IVP data from the same bulls (Table 2). Also, Marquant-Le Guienne et al. [10] and Ward et al. [26], using a small number of bulls ($n = 6$), have reported a correlation between IVP and *in vivo* bull fertility, suggesting that IVP data can be utilized to predict bull fertility.

The efficiency of a particular laboratory test to predict bull fertility is directly related to the statistical analysis methods used. To our knowledge, this is the first study to use Bayesian inference to estimate fertility in bulls. The use of Bayesian inference has been growing as an alternative statistical method, because complex problems in many fields can be solved using this method, including a limited set of data which are frequently observed in biological experiments. Moreover, the use of these models has been stimulated by the development of more sophisticated and efficient computer algorithms, like the program Winbugs 1.4 [16].

In this experiment, the estimated conception rates obtained when cleavage and blastocyst formation combined data used in the model were almost identical to the true conception rates observed for the same bulls, indicating that this is an efficient method to establish *in vivo* bull fertility estimation in commercial FTAI programs.

The estimated conception rates were still close to the previously observed true conception rates, even when cleavage rate data or blastocyst formation data were used alone. However, since the IVP methodology can lead to a great deal of variation using the same bull and in this experiment only three animals were studied, this model should be investigated for a larger number of bulls, with extremely high and low IVP and FTAI results and different *in vitro* culture conditions in order to improve its accuracy. Moreover, field source of variations, like inseminator, moment of insemination, female ciclicity, hormonal treatment, season, geographical area, and type of food should be lead in consideration in the statistic model in order to minimize the influence of external factors [29].

We conclude that Bayesian inference is a suitable statistical method to estimate the fertility of bulls using IVP data. These results are interesting because they open the possibility of developing a statistical program to estimate *in vivo* bull fertility performance, based on laboratorial data, for application in the cattle industry reducing the probability of using low-fertility bulls in AI programs.

Appendix

Bayesian inference implemented in the program Winbugs 1.4.

(A) Binomial Model:

$$Y_i \backsim \text{Binomial}\ (N_i, p_i), \quad i = 1, 2, 3, \qquad \text{(A.1)}$$

where Y_i: number of pregnant cows, N_i: total number of cows, P_i: conception rate, and i: bull.

(B) Linked functions:

$$\text{model 1}: \log\left(\frac{p_i}{1 - p_i}\right) = \alpha + \beta_1 f_{1i} + \beta_2 f_{2i}$$

$$\implies p_i = \frac{e^{\alpha + \beta_1 f_{1i} + \beta_2 f_{2i}}}{1 + e^{\alpha + \beta_1 f_{1i} + \beta_2 f_{2i}}},$$

$$\text{model 2}: \log\left(\frac{p_i}{1 - p_i}\right) = \alpha + \beta_1 f_{1i}$$

$$\implies p_i = \frac{e^{\alpha + \beta_1 f_{1i}}}{1 + e^{\alpha + \beta_1 f_{1i}}},$$

$$\text{model 3}: \log\left(\frac{p_i}{1 - p_i}\right) = \alpha + \beta_2 f_{2i}$$

$$\implies p_i = \frac{e^{\alpha + \beta_2 f_{2i}}}{1 + e^{\alpha + \beta_2 f_{2i}}}, \tag{A.2}$$

where α, β_1 and β_2 are constants calculated in program Winbugs 1.4 through MCMC computer algorithms to establish the predictive model. Cleavage data (f_{1i}) and Blastocyst formation data (f_{2i}) that were provided to the program Winbugs 1.4 to estimate conception rates (p_i).

(C) Predictive models obtained using the program Winbugs 1.4:

$$\text{model 1}: p_{io} = \frac{e^{1.0 - 3.23 f_{1i} + 13.39 f_{2i}}}{1 + e^{1.0 - 3.23 f_{1i} + 13.39 f_{2i}}},$$

$$\text{model 2}: p_{io} = \frac{e^{-0.47 + 1.1 f_{1i}}}{1 + e^{-0.47 + 1.1 f_{1i}}}, \tag{A.3}$$

$$\text{model 3}: p_{io} = \frac{e^{-1.01 + 4.87 f_{2i}}}{1 + e^{-1.01 + 4.87 f_{2i}}}.$$

Model 1: estimation of bull fertility based on combined data of cleavage and blastocyst formation. *Model 2:* estimation of bull fertility based on cleavage rates only. *Model 3:* estimation of bull fertility based on blastocyst formation rates only. f_{1i}: cleavage rate provided; f_{2i}: blastocyst formation rate provided; p_{io}: estimated bull fertility.

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