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Abstract

Sixty-seven candidate dams were used to study the effect of surrogate genotype, parity, embryo transfer phase and hormone administration method on nine animal parameters. Six cattle genotypes, six parities and two hormonal methods were evaluated. In phase I, candidates were injected with 3ml of Estrumate®. Heat expression was then detected followed by two injections 12-hr apart of the same hormone. Standing heat was observed and individuals with good sized corpus luteum were used for embryo transfer (ET). In phase II and III, CRESTAR® ear implants were placed in each candidate. Implant removal was followed by Estrumate® injection. Standing heat was later observed followed by ET. Surrogate genotype influenced BCS (P<0.001), ovary grade (P<0.05), duration in hours between the final Estrumate treatment and standing heat expression (P<0.001) and ET status (P<0.05). Ankole × Jersey crossbreds gave the shortest mean duration between synchronisation and heat (51.0 hr.) followed by Ankole × Sahiwal crossbreds (61.4 hr.). Parity, ET phase and hormone administration method did not affect the assessed parameters. Though some of the surrogates returned on heat, they sustained their pregnancies. Pregnancy diagnosis at 3 months showed a 41% of the ET surrogates successfully pregnant. ET status strongly correlated with ovary grade (r = 0.51) and CL presence (r = 0.62). Screening of candidate surrogates should therefore mainly focus on genotype.

Key words: Reproduction, embryo transfer, estrus synchronisation, cattle genetics, Rwanda.
Facators Affecting Suitability of Surrogate Dams for ...

Abbreviations

ARTs: Assisted reproductive technologies
BCS: Body Condition Score
ET: Embryo transfer

Introduction

Embryo transfer, the process by which an embryo is collected (flushed) from one female (the donor) and transferred to another female (the surrogate/recipient) to complete the gestation period, has been around for a long time. Initially done 120 years ago in a rabbit (Hasler, 2004), it only became successful in cattle (Willett et al., 1951), just sixty years ago. Despite being almost a commonplace technology in the developed world to date, adoption of embryo transfer in developing countries is still limited. To this end, the biggest limitation has been the slow development of the human skill to perform the practice and the general lack of superior donor cows. On the technological front, developments in embryo transfer technology including embryo sexing (Anderson, 1987); gene transfer (Land and Wilmut, 1987) and vitrification, a mode of preserving the embryos (Nedambale, 2005), have secured the role of this technology.

Coupled with the need to intensify livestock production in many developing countries is the need to keep high performing cattle herds. Intensification is being promoted in Rwanda mainly due to the limitation of small land sizes, low agricultural productivity and high human population density. Embryo transfer technology can adequately be used to cause rapid genetic change and build herds of elite/pedigree cattle without necessarily worrying about live animal import/export bureaucracy, and proliferation of trans-boundary livestock diseases.

Across the world, the main impetus for development of embryo transfer technology has largely been the need to overcome barriers to importation of disease agents when live animals or semen are introduced (Stringfellow, 1985). In cattle, routine embryo transfer procedures do not spread most of the infectious disease organisms (Hare, 1986), hence its suitability as an engine for driving the livestock genetic change campaign. Embryo transfer and its precursor, multiple ovulation have majorly contributed to optimisation of genetic improvement of cattle, and to some extent, in other species such as the pig, buffalo and dog. However, most MOET schemes require one or a few large nucleus herds, such that the genetic improvement that would result from a particular breeding programme would be disseminated to the general population by artificial insemination, embryo transfer, or by use of young bulls in natural mating (FAO, 1991). Due to technical, managerial and chronic financial incapacity in developing countries, it will take a much longer time for such large nuclei to be established and managed well, to fully operationalize multiple ovulation and embryo transfer (MOET) technology. In the meantime, reliance on embryo transfer (ET) alone, will at least jump start the process. Interestingly, the term “multiple ovulation and embryo transfer” was coined less than three decades ago by Nicholas and Smith (Nicholas and Smith, 1983), though the technology had been around for a much longer duration as already observed.

Currently, the government of Rwanda has been spearheading a national campaign to enhance the productivity of cattle in the country. The one cow per poor family or “Girinka” scheme, in which an in calf heifer/cow of dairy pedigree is given to a poor household was proposed in 2006 (GoR, 2006) and is currently operational, targeting a total of 600,000 households. Coupled with this was the development of the dairy sector master plan (MINAGRI, 2009), and the School Milk Programme (MINECOFIN, 2009). These grand plans are all relying on a high performing dairy sector, whose bench mark are elite dairy cows. The strategies so far put in place include importation of grade cows from within the East African region and bulls from South Africa. However, to avoid continuous importation of these grade animals to meet the set targets, there is need to strengthen capacity in assisted reproductive technologies (ARTs), such as artificial insemination, estrus synchronisation and sharp heat detection. In addition, the novel technology of multiple ovulation and embryo transfer will have to be domesticated in Rwanda so as to be able to rapidly multiply the combined gain from introduced elite sires and dams. For such efforts to be well grounded, it is prudent to initially evaluate the suitability of commercially available embryo stock,
and honed in the skills of Rwandan ART technicians. In this regard, evaluation of protocols for conducting embryo transfer would be a good starting point. It was in line with these issues therefore that we proposed to study the factors that affect the suitability of embryo transfer candidate surrogates in Rwanda.

Materials and Methods

Study Area
This study was conducted at Songa Animal Genetic Improvement Station (2° 24’ S, 29° 46’ E), of the Rwanda Institute of Agricultural Research (recently renamed Rwanda Agriculture Board), located in Southern Rwanda. The station is situated in the mid-altitude zone (1500-1600 m a.s.l.) of the country; with average annual temperature varying between 22°C and 29°C; average annual rainfall of 1087 mm and relative humidity of 77%. The rainfall pattern is bimodal, with short rains coming between September and December and long rains extending from March through May. The dry season runs between June and August. The rain is heavy in April and May, and decreases gradually until October. The natural vegetation consists of natural grass composed of Brachiaria decumbens and Themeda spp. Also available are planted multipurpose trees like Calliandra calothyrsus and Leucaena leucocephala; and grass fodder, Pennisetum purpureum. The soils are of two main types, namely clay sandy on the hills and typical clay in the marshland.

Study animals, embryos and experimental procedure
Candidate surrogate cows selected on the basis of body condition were part of the 600-strong cattle herd at Songa station in Rwanda. A total of 67 animals were used for the ET trial in three phases: I, II and III. Seventeen grade Friesian embryos collected under standard procedures were procured for this trial. In phase I, 20 animals were injected with 3 ml Estrumate® (cloprostenol sodium, Intervet, Summit, NJ). Heat detection was then conducted between 11th and 14th March of 2011. A second administration of Estrumate® was done seven days after the heat detection and repeated 12 hours later. The time for the new standing heat was recorded and responding animals prepared for ET. Heat was not observed in one individual; while another aborted. Later, the candidates were examined for corpus luteum condition; only eight individuals were found to qualify for ET and were subsequently used. However, four of the ET surrogates came back on heat between two and six weeks later.

In ET phase II initiated 11 days after phase I, a total of 38 cows/heifers were examined for ovary and body condition, and fifteen individuals were selected. In this phase, the hormonal implant method was used to synchronise the surrogates. Hormonal implants (CRESTAR®, Intervet, Pune, India) were placed in one of the ears of each candidate. Injection of multivitamin was also done on the same day. The removal of the implants was staggered over three days so as to spread the dates for the ET activity. Immediately after removal of the implants, Estrumate® injections were given to 14 of the cows, excluding one that had lost its implant. Standing heat was observed in 11 of the 15 cows, and time of heat was recorded. After checking the ovary status on the ovulation day, nine cows were chosen for ET. However, due to bad corpus luteum condition in four of the animals, only five (5) were used for ET in phase II.

Selection of cows for the ET phase III was done one month after the phase II and thereafter, the animals were synchronised with the hormonal implant method. The implants were then removed on day 7, followed by ovary examination on day 9. ET was thereafter performed on four cows on 1st and 2nd June 2011. Pregnancy diagnosis was conducted on day 40 for eight cows (ET phase I). A second PD was done on day 140. The first calf, a female (Figure 1a) was dropped 275 days after ET.

All animals were handled according to official guidelines for the safe care and handling of experimental animals.

Study Design
The study used a completely randomised design, with four main factors, namely: genotype, parity, embryo transfer phase and hormone administration method. The six cattle genotypes used were: pure Ankole (AA), Ankole × Friesian (AF) cross, Ankole × Jersey (AJ) cross, Ankole ×
Sahiwal (AS) cross, Ankole × Sahiwal × Jersey (ASJ) cross and an unclear Friesian crossbreed (EPF). The candidates were heifers (parity zero), or cows of parity between 1 and 3. Embryo transfer was done in three phases, I, II and III separated by a two month period. Hormone administration for synchronisation was done either by giving three Estrumate injections, or using hormonal implants and then using a terminal Estrumate treatment.

### Statistical Analysis

Data analysis was performed using Statistical Analysis Systems, Ver. 9.1.3 (SAS, 2004). Parameters were assessed to generate least square means using general linear models, with genotype, parity, ET phase and hormone administration method as fixed effects. The model used was:

\[
y = \mu + G_i + P_j + E_k + H_l + e_{ijklm} \sim N(0, \sigma_e^2)
\]

Where: \(y\) is the observation of the parameter for genotype \(i\), of parity \(j\), in ET phase \(k\) and for hormone administration method \(l\). \(\mu\) is the overall mean, \(G_i\) the effect of genotype \((i = 6)\), \(P_j\) the parity effect \((j = 4)\), \(E_k\) the effect of ET phase \((k = 3)\), \(H_l\) the effect of hormone administration method \((l = 2)\), \(e_{ijklm}\) the random effect on the parameter, independently and identically distributed with mean \(= 0\) and variance \(= \sigma_e^2\).

Correlation coefficients were computed to determine the association between cow parity, body condition score (BCS), corpus luteum (CL) condition before synchronisation, ovary grade, hours from 1<sup>st</sup> Estrumate treatment to heat expression, CL presence after synchronisation, hours from 3<sup>rd</sup> Estrumate treatment to standing heat, embryo transfer (ET) status and pregnancy diagnosis (PD) status.

### Results and Discussion

The effects of surrogate genotype, parity, embryo transfer (ET) phase and hormone administration method were the focus of this study. Several factors have been identified to affect pregnancy rates following embryo transfer in cattle (Rao et al., 2011) and other species (Misra et al., 1999). In this study, genotype significantly influenced body condition score \((P<0.001)\), ovary grade \((P<0.05)\), duration in hours between the final Estrumate treatment and standing heat expression \((P<0.001)\) and ET status \((P<0.05)\). While Ankole × Friesian crossbreds had relatively good body condition score and ovary grade (Table 1), they did not respond to hormonal treatment, contrary to our expectations and past studies. On the other hand, Ankole × Jersey crosses gave the best responses expected of good ET surrogates. The bovine surrogate female has been found to have less influence on the performance of embryo transfer calves in some earlier studies. However, elsewhere, differences found between beef and dairy dams (Humes et al., 1987) link to differences in maternal environment and lead to transplanted embryos not being able to express their genetic potential in case recipient cows are from breeds of low milking ability and mature size. Maternal genotype effects in other mammalian species such as the mouse (Cowley et al., 1989) and rabbit (Mocé et al., 2004) are even more succinct.

The hormone administration method only had a significant influence on ovary grade \((P<0.001)\) and ET status \((P<0.05)\). Our results imply that surrogates responded better to use of ear implants compared to the three Estrumate injections (Table 2). The ear implant procedure comes with the additional advantage of stressing the animal less since the animal is confined into the crush less times, and is also associated with a lower cost since less injectable hormone is administered. A related study comparing three approaches for synchronisations of ovulation in Bos indicus–influenced cattle (Williams et al., 2002) reported that implants were better than multiple hormone injections. Manipulation and control of the estrus cycle in pasture–based dairy cows (Cavaliere et al., 2006), found, just like in our study, that protocols which use progesterone releasing inserts were more effective at inducing estrus.

Though the allocation of candidate surrogates to the ET phase was random, results show that significant differences were present in the body condition score of the animals (Table 2). The only plausible explanation regards the nutritional quality of available material over the study period, though usually nutritional imbalances do not show immediately before or after the stress period. Across the ET phases, ovary grade took a trend opposite to
that of body condition score. An animal with a good body condition should be in the best state to possess ovaries in the best grade; however, this sometimes is confounded by other factors which come into play. Surrogates seem to have had an even chance to receive an embryo, irrespective of ET phase, no wonder; pregnancy diagnosis status did not significantly differ across ET phases (Table 2).

The summary statistics for the various parameters are presented in Table 3. When scored on a 1–5 hedonic scale, generally the animals had good body condition scores. Our findings are in agreement with practices of screening candidate surrogates, their synchronization and embryo transfer elsewhere. The screening of recipients in this study was based on ovary grade, parity and body condition. Most of the recipients chosen had a good CL and good body condition grade. Earlier reports (Cavalieri et al., 2006) found that a good recipient must be on a proper plane of nutrition, with a body condition score of 6 for beef cows and dairy body condition score of 3 to 4 for dairy breed recipients. It is also normal practice to ensure that the cows are on a sound herd health program. Other reports (Pradhan et al., 2008; Tervit et al., 1977) indicated that season and parity are also key players in success of MOET.

### Table 1: Least square means for effect of surrogate genotype

<table>
<thead>
<tr>
<th>Surrogate genotype (n = 67)</th>
<th>Body condition score</th>
<th>Ovary grade</th>
<th>Duration II (hours)</th>
<th>CL grade</th>
<th>ET Status</th>
<th>PD status</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>2.0a</td>
<td>2.69a</td>
<td>68.8a</td>
<td>2.0</td>
<td>2.0a</td>
<td>-</td>
</tr>
<tr>
<td>AF</td>
<td>3.0b</td>
<td>3.28b</td>
<td>140b</td>
<td>2.0</td>
<td>2.0b</td>
<td>-</td>
</tr>
<tr>
<td>AJ</td>
<td>3.0b</td>
<td>2.50a</td>
<td>51.0a</td>
<td>2.0</td>
<td>1.8a</td>
<td>2.0</td>
</tr>
<tr>
<td>AS</td>
<td>3.1b</td>
<td>2.06a</td>
<td>61.4a</td>
<td>1.5</td>
<td>1.6b</td>
<td>1.6</td>
</tr>
<tr>
<td>ASJ</td>
<td>2.7c</td>
<td>2.33a</td>
<td>61.2a</td>
<td>1.6</td>
<td>1.6b</td>
<td>1.0</td>
</tr>
<tr>
<td>EPF</td>
<td>-</td>
<td>4.00bc</td>
<td>60.2a</td>
<td>1.0</td>
<td>1.3b</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Means within the same column but with different superscripts differ significantly ($P < 0.05$)

1Hours between 3rd estrumate treatment/implant removal and standing heat

### Table 2: Least square means for effect of hormonal treatment method and embryo transfer phase

<table>
<thead>
<tr>
<th>Treatment method (n = 67)</th>
<th>Body condition score</th>
<th>Ovary grade</th>
<th>Duration II (hours)</th>
<th>CL grade</th>
<th>ET Status</th>
<th>PD status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrumate Injections</td>
<td>3.03</td>
<td>1.56a</td>
<td>66.8</td>
<td>1.9</td>
<td>1.55a</td>
<td>1.37</td>
</tr>
<tr>
<td>Ear Implant</td>
<td>2.79</td>
<td>2.82b</td>
<td>73.6</td>
<td>1.4</td>
<td>1.80b</td>
<td>1.77</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ET Phase (n = 67)</th>
<th>Body condition score</th>
<th>Ovary grade</th>
<th>Duration II (hours)</th>
<th>CL grade</th>
<th>ET Status</th>
<th>PD status</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3.03a</td>
<td>1.56a</td>
<td>66.8</td>
<td>1.70</td>
<td>1.55a</td>
<td>1.37</td>
</tr>
<tr>
<td>II</td>
<td>2.53b</td>
<td>2.89b</td>
<td>73.6</td>
<td>1.60</td>
<td>1.86b</td>
<td>1.80</td>
</tr>
<tr>
<td>III</td>
<td>3.22c</td>
<td>2.50b</td>
<td>-</td>
<td>1.33</td>
<td>1.55a</td>
<td>1.75</td>
</tr>
</tbody>
</table>

Means within the same column but with different superscripts differ significantly ($P < 0.05$)

1Hours between 3rd estrumate treatment / implant removal and standing heat

Embryo transfer was done after the ovaries of all the candidates had been examined to determine which ovary had a corpus luteum because the embryo must be placed in the uterine horn of the embryo that had ovulated and hence had a corpus luteum. The embryos were transferred into the left or right horn depending on ovulation site using the broad ligament, and hence avoiding touching uterus as much as possible to avoid prostaglandin releases that reduce pregnancy rates. Transferring embryos ipsilateral or contralateral to the functional corpus luteum has been found to lead to 54% and 39% pregnancy rates respectively (Tervit et al., 1977). Recent studies in India (Rao et al., 2011) have also found that site of embryo deposition and grade/quality of transfer significantly influenced pregnancy rate in over half of the recipient cows. Therefore prudence must be exercised in identifying the true ovary with the active corpus luteum. What we found odd was that many of the surrogates carrying the embryos returned on heat in the
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aftermath of the embryo implantation (Table 3). While unexpected to occur to such an extent, it has been reported to occasionally occur, especially when assisted reproductive technologies such as use of fertility enhancement hormone administration is done. The transfer of embryos can be utilised to determine the suitability of recipients for maintaining pregnancy following fertilisation and early embryo development (Hasler, 2006) and is a way of assessing infertility in dairy cattle which is increasingly becoming widespread in dairy herding. Success rate for pregnancy is affected by the number of embryos transferred (Heyman et al., 1985); hence we can suppose that more embryos transferred implies more experienced and confident personnel.

Correlation coefficients between ovary grade and hours from 1st Estrumate treatment to heat expression, ovary grade and ET status, CL presence and ET status, CL presence and hours from 3rd Estrumate treatment to standing heat showed interesting relationships. We found that ovary grade significantly \( (P = 0.044) \) correlated with hours from 1st Estrumate treatment to heat expression \( (r = 0.54) \). Duration II strongly correlated \( (r = 0.41, P = 0.029) \) with corpus luteum grade. Corpus luteum grade also correlated strongly with ET status \( (r = 0.62, P = 0.0001) \). These results support our earlier assertion that the initial ovary grade is as important as the corpus luteum condition after synchronisation, in selecting the best surrogates for embryo transfer. A computation of pregnancy rates after ET for our study gives 41%, and two calves from the confirmed pregnancies have so far been delivered (Figure 1 & 2). According to Hasler (2004), in commercial situations, pregnancy rates in cattle can be as high as 70%. Therefore, for an experimental study, such as the one reported in this paper, critique of our rate should be with caution. Other key factors to include in ensuring ET success are: corpus luteum characteristics, and progesterone concentration levels (Spell et al., 2001). Corpus luteum diameter or luteal tissue volume has been found to strongly correlate with plasma progesterone concentration (Spell et al., 2001); hence, the bigger the corpus luteum, the higher the hormone level and the better the chance that a pregnancy will be sustained.

**Table 3:** Summary statistics for the parameters studied.

<table>
<thead>
<tr>
<th>Variable (n = 67)</th>
<th>Mean</th>
<th>s.e</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCS(^1)</td>
<td>2.89</td>
<td>0.07</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Ovary with CL(^2)</td>
<td>1.49</td>
<td>0.17</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>CL Condition(^3)</td>
<td>2.82</td>
<td>0.18</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Ovary Grade(^4)</td>
<td>2.71</td>
<td>0.12</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Hours between 1st Estrumate to heat</td>
<td>88.10</td>
<td>10.60</td>
<td>20</td>
<td>&gt;140</td>
</tr>
<tr>
<td>Hours between 3rd Estrumate to standing heat</td>
<td>69.80</td>
<td>5.80</td>
<td>27</td>
<td>&gt;140</td>
</tr>
<tr>
<td>CL grade(^4)</td>
<td>1.58</td>
<td>0.12</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>ET status(^2)</td>
<td>1.73</td>
<td>0.05</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Days between ET and return to heat</td>
<td>34.38</td>
<td>6.46</td>
<td>11</td>
<td>84</td>
</tr>
<tr>
<td>PD status(^2)</td>
<td>1.59</td>
<td>0.12</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^1\)On a 1–5 hedonic scale; \(^2\)Presence = 1, Absence = 2; \(^3\)On a 1–3 hedonic scale; \(^4\)On a 1–5 hedonic scale.

**Fig. 1:** a) Female calf born of Jersey × Ankole crossbred dam by embryo transfer on Dec. 27\(^{th}\) 2011. b) Male calf born of Sahiwal × Ankole crossbred dam by embryo transfer.
Conclusion

The performance of surrogate dams in our study indicates that the main factors were surrogate genotype and hormone treatment method. However, the contribution of secondary factors such as the human factor, the embryo factors and animal factors not addressed in this study need to be quantified to enhance the overall success rates. We recommend that selection of surrogates for embryo transfer should primarily focus on the Ankole × Jersey crossbred cows. The implant technique should be preferred to the multiple hormone treatments. A broader study, using more individuals per genotype, and balancing for all the factors is recommended to confirm our findings.

Acknowledgements

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