GENETIC PATERNITY TEST FOR PIGLETS GENERATED BY HETEROSPERMIC CERVICAL AND POST-CERVICAL ARTIFICIAL INSEMINATION

[*Determinação genética da paternidade em leitões nascidos de inseminação artificial heterospérmica cervical e pós-cervical*]

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ABSTRACT - Post-cervical artificial insemination (AI) with reduced sperm concentration allows the use of semen from few boars on a high number of females, but the common use of pooled semen in commercial AI programs masks the subfertility of individual boars. This study compared the reproductive performance of post-cervical and cervical heterospermic AI, in commercial farm conditions and through paternity tests. Each AI method was conducted in two groups of 150 sows. Sperm concentration was 3.0 x 10^9/85mL for cervical AI and 1.5 x 10^9/60mL for post-cervical AI. Conception and farrowing rates for post-cervical AI (90.7% and 85.3%, respectively) were lower (P<0.05) than for cervical AI (98.7% and 94.7%, respectively). Total litter size did not differ (P>0.05) for post-cervical (12.8 ± 0.3) and cervical AI (13.5 ± 0.3), but it was greater (P<0.05) for parity-two females than for those having 3-5 parities. The paternity test used nine microsatellite markers to genotype 300 piglets from 25 litters, but paternity exclusion was achieved for only 95 piglets, which did not differentiate boars across AI methods.

Keywords: Post-cervical AI, paternity test, micro-satellites, swine.

RESUMO - A inseminação artificial intra-uterina (IAIC) com reduzida concentração de espermatozóides por dose permite que o sêmen de um menor número de reprodutores seja usado em um maior número de fêmeas. Porém, o uso freqüente de *pools* de sêmen de dois ou mais machos na inseminação heterospérmica pode mascarar o baixo desempenho de alguns reprodutores. O objetivo deste estudo foi comparar o desempenho reprodutivo da IAIU com a inseminação artificial intracervical (IAIC), usando amostras heterospérmicas de sêmen, em condições de rotina de campo e através de teste de paternidade. A IAIC foi feita com 3,0 x 10^9 espermatozóides/85mL e a IAIU foi feita com 1,5 x 10^9 espermatozóides/60mL, ambas em grupos de 150 fêmeas. A IAIU apresentou taxas menores (P<0,05) de concepção (90,7%) e parição (85,3%) do que a IAIC (98,7% e 94,7%, respectivamente). O tamanho total da leitegada não diferiu (P>0,05) entre IAIC (13,7 ± 0,3) e IAIU (13,0 ± 0,3), mas foi mais alto nas fêmeas de segundo parto do que nas com 3-5 partos (P<0,05). O teste de paternidade usou nove microssatélites para a genotipagem de 25 leitegadas, totalizando 300 leitões, havendo exclusão de paternidade somente em 95 leitões, o que não permitiu diferenciação entre os machos, dentro das técnicas de IA.

Palavras-Chave: Inseminação artificial intra-uterina, teste de paternidade, microssatélites, suínos.

INTRODUCTION

The use of artificial insemination (AI) has been intensified in commercial swine farms, due to benefits on genetic improvement and health status. Conventionally, AI in swine is conducted by depositing cooled semen inside the cervix, at a concentration of nearly 3 x 10^9 viable sperm cells (Flowers & Esbenshade, 1993), two to three times during the estrus (Xue et al., 1998a; b; Corrêa et al., 2002). Such sperm concentration is much higher than what would be necessary for fertilization (Rath,

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2002), which can be justified by the fact that female swine have prolonged and highly variable estrus duration (Weitze et al., 1994; Kemp & Soede, 1996; Lucia et al., 1999) and ovulate on the final third part of the estrus, although the timing of ovulation is difficult to detect, unless ultrasound is used (Weitze et al., 1994; Kemp & Soede, 1996; Alvarenga et al., 2006). Thus, with cervical AI, spermatozoa must cross the uterus and reach the oviduct to fertilize oocytes (Rath, 2002).

The use of AI in routine farm conditions allows an increase in the sow:boar ratio (Flowers & Esbenshade, 1993). Such benefit is further maximized with post-cervical AI, with nonsurgical sperm deposition into the uterine body at low concentrations per dose, such as 2-1 x 10^9 (Watson & Behan, 2002; Serret et al., 2006) and 0.5 x 10^9 spermatozoa (Mezalira et al., 2005). Thus, individual boars would increase their impact on herd reproductive performance. Post-cervical AI has provided results similar to those obtained with cervical AI in some studies (Watson & Behan, 2002; Mezalira et al., 2005), but other studies conducted under routine farm conditions reported inferior performance with post-cervical AI (Rozeboom et al., 2004; Roberts & Bilkei, 2005; Serret et al., 2006).

As fewer boars are used in post-cervical AI, precise methods would be necessary to select those having the greatest potential fertility. *In vitro* evaluations of semen quality based on sperm motility, vigor and morphology are commonly used, although they may not be truly associated with fertility (Linford et al., 1976; Xu et al., 1998), likely due to differences across boars (Mezalira et al., 2005) and to their differential response to distinct methods of fertility estimation (Popwell & Flowers, 2004). Thus, heterospermic AI is commonly used at farm level by pooling ejaculates from different boars, to dilute the impact of boar differences (Dziuk, 1996), even with post-cervical AI (Serret et al., 2006). So, distinct boars would contribute differently for the paternity of the litters. Such contribution could be identified by paternity tests using microsatellites markers, which are easy to genotype and abundantly available in the genome of mammals. However, such technique has been used in swine only eventually (Stahlberg et al., 2000; Nechtelberger et al., 2001; Putnová et al., 2003), likely because the genetic improvement in swine follows a population approach and thus the commercial value of individuals is not high. This study compares conception and farrowing rates and total litter size obtained with heterospermic cervical and post-cervical AI at farm level and uses paternity tests to compare the contribution of individual boars for the paternity of a sample of litters.

MATERIALS AND METHODS

This was an observational trial conducted on a commercial farm having a 1,000 female inventory, located in the Rio Grande do Sul state, Brazil, during four months. Six boars identified as A, B, C, D, E and F, from the same genetic basis and having known fertility were the semen donors. The boars were housed in an AI stud, in individual crates. Semen collections were performed using the gloved-hand technique (Bearden & Fuquay, 1997), using a plastic glass protected by an isothermic vacuum bottle covered with gauze, to separate the gel-rich fraction. After collection, sperm motility was evaluated by optical microscopy, at 200x magnification. Only ejaculates having motility equal or higher than 80% were used. Sperm concentration was measured in a spectrophotometer. Semen from randomly chosen pairs of boars was pooled in two treatments: cervical AI, 3.0 x 10^9 spermatozoa in 85mL; and post-cervical AI with 1.5 x 10^9 spermatozoa in 60mL. Semen pools were diluted in a long-term extender (X-Cell®, IMV Technologies, São Paulo-SP, Brazil) and stored in plastic blisters.

After an average lactation length of 21d, three hundred weaned females from the same genetic basis and with parity between two to five were housed in individual stalls and allocated to the two treatments (n = 150, each). The females were paired according to: parity; body condition; previous lactation length and weaning-to-estrus interval (both having at most a two-day difference); and previous total litter size (categorized in 9-11, 11-14 and more then 14 piglets). Estrus detection was conducted twice daily, at 8:00 and 16:30h, through back pressure, by a technician in the presence of a sexually mature boar. In both groups, females were inseminated three times during the estrus, at 12, 24 and 36h post-detection. After the AI, the females were kept in individual crates up to 100d of gestation. Both cervical and post-cervical AI were conducted with disposable plastic catheters. Pregnancy diagnosis was done 21d after the AI by transcutaneous ultrasonography, with a 5MHz probe (Tringa 50S, Pie Medical®).

Conception and farrowing rates were calculated as described by Dial et al. (1992) and compared across treatments through the chi-square test. Data about total litter size were obtained from the farm's records and compared across treatments through analysis of

variance, with comparison of means through the least significant difference method. The effects of parity and potential interactions were also tested. All statistical analyses were conducted using STATISTIX® (2003).

From each boar, 10mL blood samples were collected from the jugular vein in vacutainers with 50µL of EDTA. After collection, blood samples were stored in thermal boxes with ice and transported to the Biotechnology Center of the Universidade Federal Pelotas (UFPel). Only five boars provided blood samples because Boar B died immediately after the beginning of the farm trial, before any sample could have been collected. For DNA extraction, a 500µL blood samples were put into a vacutainer tube with 5mL of EDTA, which was placed in a 1.5mL eppendorf tube containing 1mL of a hemolytic buffer (Solution A), including: 250µl of 1M Tris-Cl, pH 7.6 (Vetec Química, Rio de Janeiro-RJ, Brazil); 250µl of 0.5M $MgCl_2$ (Synth, Diadema-SP, Brazil); 50µl of 5M NaCl (Synth, Diadema-SP, Brazil); and pure H_2O up to a total of 25mL. After homogenization, the solution was centrifuged at 14,000RPM for 10min. The supernatant was discharged and the resulting leukocyte pellet was re-suspended in 1mL of the Solution A and centrifuged again at 14,000RPM for 10min. Such procedure was repeated nearly 10 times to assure that no residual eritrotrocytes remained in the samples. After that, the pellet was re-suspended in 498µl of a detergent buffer (Solution B), containing: 5µl of 1M Tris-Cl, pH 8.0; 10µl of 5M NaCl; 10µl of 0.5M EDTA, pH 8.0; 12.5µl of 20% SDS (Fischer Cientific, New Jersey, NY, USA); and pure H_2O up to a total of 500µl. Then, 2µl of K Proteinase (Gibco-Invitrogen, Grand Island, NY, USA) was added to the solution. After homogenization, samples were incubated at 55ºC, during 4-6h or overnight, until the pellet was dissolved. Thereafter, 500µl of Solution B and 316µl of 5M NaCl were added and samples were centrifuged at 14,000RPM for 15min. Then, 500µl of the supernatant was transferred to a sterile eppendorf tube, in which 1mL of 100% ethanol was added. After homogenization, the content was centrifuged at 14,000RPM for 15min. After discharge of the supernatant, 200µl of frozen 70% ethanol was added and samples were centrifuged at 14,000RPM for 15min. The resulting pellet was re-suspended in 250µl of TE buffer (10mM 10mL of 1.0M TRIS (Vetec Química, Rio de Janeiro-RJ, Brazil), pH 8.0; 1mM 2mL of 0.5M EDTA; 121.1g TRIS; and 700mL distilled H_2O for a total of 1.0L). Then, samples were stored in a freezer.

After farrowing, two thirds of the tails were docked from the piglets born during the farm trial. The tails were put in 100mL plastic bags containing 25mL of 70% ethanol, transported to the laboratory inside thermal boxes and stored at 5ºC. From all litters farrowed, twenty five having complete information about total born litter size and all tails correctly identified were randomly selected for the paternity test. After removal from ethanol, a fragment of each tail was cut, identified, covered with paper and incubated at 37ºC for 10min, for DNA extraction. After incubation, a sample of 30mg of dry tissue had its skin removed, and the muscle was put in a 1.5mL eppendorf tube, with 500µl of cetyltrimethyl ammonium bromide buffer (CTAB) (Sigma Chemical Company, St. Louis, MO, USA): 100mL of 1M TRIS, pH 8.0; 280mL of 5M NaCl, 40mL of 0.5M EDTA; and 20g of CTAB, for a total of 1L. The content was put in water bath at 65ºC and crushed. After 1h in water bath, samples received 2µL of 2% ß-mercaptoetanol (Sigma Chemical Company, St. Louis, MO, USA) and were kept at room temperature for 30min. After that period, the samples received 4µL of K Proteinase and were put in water bath at 45ºC during 1h, under homogenization. After addition of 1µL of RNAse (Gibco-Invitrogen, Grand Island, NY, USA), the water bath temperature was raised to 55ºC and kept overnight for nearly 16h, under homogenization. After that, phenol-clorophorm (Synth, Diadema-SP, Brazil) was added and the content was centrifuged at 13,000RPM for 10min. The water-like phase, formed in the upper part of the tube, was put in an eppendorf tube with 100% isopropanol (Synth, Diadema-SP, Brazil). After 1h at -20ºC, samples were centrifuged at 13,000RPM for 6min. The supernatant was discharged and the eppendorf tube was left open in incubation at 45ºC during 30min, for elimination of all ethanol content. Then, the pellet was re-suspended in 350µl of 1.0M NaCl, homogenized and subsequently centrifuged at 13,000RPM for 13min. The supernatant was discharged and 500µL of 70% ethanol was added, to remove the salt content. The samples were centrifuged at 13,000RPM for 3min and left to dry until the ethanol odor disappeared. The pellet was re-suspended in 50µL of TE buffer and put in a 0.8% agarosis gel. After extraction, DNA samples were stored at -20ºC.

Paternity tests were done with nine microsatellite markers (SO090, SO101, SO155, SO355, SO386, SW24, SW240, SW857 and SW951) indicated by International Society for Animal Genetics (ISAG), previously used in swine (Nechtelberger et al.,

2001; Putnová et al., 2003). The forward primer of each marker was conjugated to one fluorescent sond, according to the size of its fragment, to allow genotyping of the PCR's product. Two multiplex were formed from the selected markers, distributed according to the expected size of the fragments (Table 1). The PCR was conducted as described by Nechtelberger et al. (2001) and amplified in a thermo cycler (Eppendorf, São Paulo-SP, Brazil). To verify DNA integrity, all samples were analyzed by electrophoresis in a 0.8% agarosis gel in a TRIS-borate-EDTA buffer stained with Etidhium Bromate (Gibco-Invitrogen, Grand Island, NY, USA). The DNA was quantified in 12 samples randomly collected during the extraction. The PCR's product was genotyped in an ABI 377 Genetic Analyzer® (Applied Biosystems) using the GeneScan® software (Eurofins Scientific). The results were expressed by the percent of paternity exclusion per litter.

RESULTS

Overall conception and farrowing rates were equal to 94.7% e 90.0%, respectively. Conception rate for cervical AI (98.7%) was higher ($P<0.05$) than for post-cervical AI (90.7%). Farrowing rate was also higher ($P<0.05$) for cervical (94.7%) than for post-cervical AI (85.3%). However, none of those rates was influenced by parity ($P>0.05$). Conception rates were 96.7% for parity-two females and 93.8% for those having 3-5 parities, whereas farrowing rates for parity-two and parity 3-5 females were 90.2% and to 89.9%, respectively.

Mean total litter size for all inseminated females was 13.2 ± 3.0. Total litter size did not differ ($P>0.05$) between cervical (13.5 ± 0.3) and post-cervical AI (12.8 ± 0.3). However, total litter size for females having 3-5 parities (13.6 ± 0.2) was higher ($P<0.05$) than for parity-two females (12.7 ± 0.3). No significant treatment per parity interaction was observed ($P>0.05$).

The DNA extracted from both blood and tails had little evidence of degradation or contamination. However, only seven out of the nine microsatellite markers used in this trial could be used in the paternity test. The SO090 marker did not produce amplification on any target region of the genome, showing no evidence of alleles in the PCR product. Additionally, the SW24 marker presented monomorphic amplification, since all obtained alleles were the same for all tested individuals.

The 25 genotyped litters included 300 piglets born, with mean total litter size of 12.0 ± 2.5. Among them, 10 were generated by cervical AI (n = 129 piglets), with mean total litter size of 12.9 ± 0.8, and 15 were generated by post-cervical AI (n = 171 piglets), with mean total litter size of 11.4 ± 0.6. In the genotyped litters, no difference in total litter size was observed between AI methods ($P>0.05$). The presence of three distinct loci for each individual was used as the basis for exclusion of paternity, but that was accomplished for only 94 piglets (33.0%). In three litters, the paternity of four piglets could be attributed to both boars included in the semen pool (Table 2).

The exclusion of paternity per boar is shown in Table 3, excluding Boar B, who died prior to the blood sampling. The exclusion of paternity was similar for the two AI methods for most boars, with the exception of Boar F, for whom most piglets having exclusion of paternity were generated by cervical AI. Among the semen pools, pool 4 was the one having more genotyped piglets (n = 119). From those, 54 had their paternity identified, with predominance of Boar D over Boar F both in cervical (20 vs. 7 piglets, respectively) and post-cervical AI (26 vs. 2, respectively). In pool 5, 26 piglets were genotyped, but only 12 had their paternity identified, all sired by Boar E (6 in each AI method). Table 4 shows the alleles obtained by the genotyping of boars, which, in many cases, were equal or similar.

DISCUSSION

The overall conception and farrowing rates and total litter size observed in this study were at desirable levels, according to industry standards (Dial et al., 1992, Corrêa et al., 2002). However, post-cervical AI presented lower conception and farrowing rates than cervical AI. Total litter size did not differ across AI methods, but the 0.7 unit advantage observed for cervical AI, although not significant, may be relevant at farm level. Some studies reported no differences in performance between cervical and post-cervical AI (Watson & Behan, 2002), which favors the latter, due to the lower sperm concentration. On the other hand, other studies conducted under routine farm conditions reported inferior performance with post-cervical AI (Rozeboom et al., 2004; Roberts & Bilkei 2005; Serret et al., 2005), which probably reflects the effect of factors such as sow parity and sperm concentration. The effect of parity was also observed in this study, since parity-two females presented inferior performance. Nevertheless, it is important to highlight that conception and farrowing rates observed in this study with cervical AI were

Table 1. Loci used in each multiplex.

Locus	Multiplex/color	Place in chromosome	Fragment size
SW24	Multiplex - FAM	17	92 - 112
SW951		10q	124 - 136
SW857		14	145 -159
SO386		11q	164 - 182
SO101		7q	196 - 224
SO090		12q	243 - 253
SW240	Multiplex - HEX	2q	94 - 114
SO155		1q	148 - 164
SO355		15	245 - 271* / 241 - 269 #

*Nechtelberger et al. (2001)
#Putnová et al. (2003)

Table 2. Exclusion of paternity per litter, by semen pool, boar and artificial insemination (AI) method (CE: cervical; PC: post-cervical).

Semen pool	AI	Total born	Boars						No exclusion
			A	B	C	D	E	F	
1	CE	15	3	0	-	-	-	-	12
1	CE	17	1	0	-	-	-	-	16
1	CE	7	1	0	-	-	-	-	6
1*	CE	12	0	-	2	-	-	-	10
1	PC	7	0	0	-	-	-	-	7
1	PC	12	0	0	-	-	-	-	12
1	PC	14	2	0	-	-	-	-	12
1	PC	14	3	0	-	-	-	-	11
1	PC	11	2	0	-	-	-	-	9
2	CE	13	-	0	7	-	-	-	6
2	PC	10	-	0	5	-	-	-	5
3	CE	12	-	0	-	-	-	1	11
3	PC	11	-	0	-	-	-	0	11
4*	CE	14	-	-	-	2	-	5	7
4	CE	14	-	-	-	7	-	2	5
4	CE	13	-	-	-	11		0	2
4	PC	8	-	-	-	5	-	0	3
4	PC	14	-	-	-	1	-	0	13
4	PC	14	-	-	-	6	-	0	8
4	PC	11	-	-	-	6	-	0	5
4	PC	12	-	-	-	1		0	11
4	PC	9	-	-	-	7	-	1	1
5	CE	12	-	-	-	-	6	0	6
5*	PC	14	-	-	-	-	6	0	8
5	PC	10	-	-	-	-	1	0	9
Total		300	12	0	14	46	13	9	206

*Litters in which paternity of some piglets could be attributed to both semen donors in the pool (2: one piglet; 10: one piglet; 12: two piglets)

Table 3. Piglets having exclusion of paternity by boar and artificial insemination method.

Boar	Genotyped piglets	Artificial insemination	
		Cervical	Post-cervical
A	12	5	7
C	14	9	5
D	46	20	26
E	13	6	7
F	9	8	1
Total	94	48	46

Table 4. Alleles of the boars used in the paternity test.

Boars	SW951	SW857	SO386	SO101	SW240	SO155	SO355
A	124	151	178	202/212	94	164	247
C	124/130	153/157	186	198/212	92/94	164	247
D	124	153	178/184	212/218	92	164	247
E	124	153/155	178	212	92	152/160	247
F	122/130	155	178	212	90/98	166	249

excellent. The mean total litter size observed for distinct AI methods, considering only the genotyped litters, followed the same trend observed for all litters, with a substantial, but not significant, 1.5 unit advantage for cervical AI. However, the low exclusion of paternity did not allow the expression of significant differences in total litter size across AI methods.

Considering the 25 genotyped litters, semen pool 4 was the most commonly used, including 54 piglets with known paternity, which was nearly half of the piglets sired by that pool (49.5%). Among those, only eight were sired for Boar F (seven with cervical AI and one with post-cervical AI) and the other 46 piglets were sired by boar D (20 with cervical AI and 26 with post-cervical AI). Within the nine litters from semen pool 4, considering the total litter size and the number of genotyped piglets per boar, Boar D would certainly have advantage over Boar F in at least five litters, even if the paternity could have been excluded for all piglets. Thus, Boar D would probably have similar performance with both AI methods, but Boar F apparently presented reduced reproductive potential, especially with post-cervical AI, which would not be evident with heterospermic AI. However, due to the limited paternity identification observed in this study, such assumption is not fully supported. Boar F might also have disadvantage in comparison with Boar E in the litters from semen pool 5, since Boar E sired all the thirteen piglets with known paternity. However, only three of the genotyped litters were from that semen pool and paternity was identified for only 36% of the piglets.

Although the extracted DNA presented good quality, paternity tests presented unsatisfactory results. So, the efficiency of such tests does not depend exclusively on DNA quality and on the number of markers, but also on the amount of information provided by that material (Ron et al., 1995). If the genotyped region of the marker does not present high polymorphism, the probability of paternity misidentification increases due to false positive or false negative results (Xu et al., 2000). Thus, the reduced paternity exclusion may be due to the low polymorphism of the markers used in this study, even though they were recommended by the ISAG (Nechtelberger et al., 2001; Putnová et al., 2003). Apparently, such a recommendation would not guarantee the test's efficiency, since a study conducted in dairy cattle, with nine markers, reported higher paternity determination for the only two markers not recommended by the ISAG than for other seven that were recommended (RON et al., 1995). The reduced paternity identification could also be due to inbreeding among the boars used as semen donors, which may be common in commercial herds, but such results cannot be exclusively attributed to that factor, since monomorphism was observed in only one locus, although the alleles in the other loci presented very similar values. Also, the occurrence of that monomorphic locus may be attributed to the presence of a given gene under selection pressure, as a consequence of process of the genetic improvement conducted in that population. It is also possible that a mutation may have occurred in the micro-satellites during the formation of the gametes, which is quite common, especially in long alleles,

which could lead to the inclusion or exclusion of one or more pair of basis (Xu et al., 2000), because the micro-satellites are randomly distributed in the genome of eukaryotes and have repetitions in tandem, especially for di-nucleotides.

CONCLUSIONS

Post-cervical artificial insemination presented higher conception and farrowing rates than cervical artificial insemination. The advantage in total litter size for post-cervical artificial insemination, in both the farm trial and for the genotyped litters, was substantial, but not statistically significant. Some boars apparently are highly fertile, regardless of the technique, whereas lowly fertile boars would be less efficient with post-cervical AI. However, as the paternity identification was inefficient, further studies are necessary to support that conclusion.

REFERÊNCIAS

Alvarenga M.V.F., Bianchi I., Schmitt E., Varela Junior A.S., Calderam O., Corrêa M.N., Deschamps J.C. & Lucia T.Jr.2006. Characterization of estrus profile in female swine and its accuracy in estimating ovulation time in comparison to ultrasound diagnosis. Animal Reproduction, 3:364-369.

Bearden H.J. & Fuquay J.W. 1997. Semen collection, In:___. Applied Animal Reproduction. 4th Ed. New Jersey: Prentice Hall, pp. 147-157.

Corrêa M.N., Lucia T.Jr., Afonso J.A.B. & Deschamps J.C. 2002. Reproductive performance of early-weaned female swine according to their estrus profile and frequency of artificial insemination. Theriogenology, 58:103-112.

Dial G.D., Marsh W.E., Polson D.D. & Vaillancourt J-P. 1992. Reproductive failure: differential diagnosis. In: Leman A.D., Straw B.E., Mengeling W.L., D'Allaire S. & Taylor D.J. Diseases of swine, 7th Ed. Iowa State University Press. Ames, IA-USA. 88-137.

Dziuk P.J. 1996. Factors that influence the proportion of offspring sired by a male following heterospermic insemination. Animal Reproduction Science. 43:65-88.

Flowers W.L. & Esbenshade K.L. 1993. Optimizing management of natural and artificial matings in swine. Journal of Reproduction and Fertility (Suppl.). 48: 217-228.

Kemp B. & Soede, N.M. 1996. Relationship of weaning-to-estrus interval to timing of ovulation and fertilization in sows. Journal of Animal Science, 74: 944-949.

Linford E., Glover F.A., Bishop C. & Stewart D.L. 1976.. The relationship between semen evaluation methods and fertility in the bull. Journal of Reproduction and Fertility, 147: 283–291.

Lucia T. Jr., Correa M.N., Deschamps J.C., Peruzo I.A., Matheus J.E. & Aleixo J.A. 1999. Influence of equine chorionic gonadotropin on weaning-to-estrus interval and estrus duration in early-weaned, primiparous, female swine. Journal of Animal Science, 77: 3163-3167.

Mezalira A., Dallanora D., Bernardi M.L., Wentz, I. & Bortolozzo F.P. 2005. Influence of sperm cell dose and post-insemination backflow on reproductive performance of intrauterine inseminated sows. Reproduction in Domestic Animals, 40: 1-5.

Nechtelberger D., Kaltwasse C., Meyer J.N., Brem G., Mueller M. & Muller S. 2001. DNA microsatellite analysis for parentage control in Austrian pigs. Animal Biotechnology, 12:141-144.

Popwell J.M. & Flowers W.L. 2004. Variability in relationships between semen quality and estimates of in vivo and in vitro fertility in boars. Animal Reproduction Science, 81:97–113.

Putnová L., Knoll A., Dvorak V. & Dvorak J. 2003. A novel porcine microsatellite panel for the identification of individuals and parentage control in the Czech Republic. Czech Journal of Science, 48: 307-314.

Rath D. 2002. Low dose insemination in the sow: a review. Reproduction in Domestic Animals, 37: 201-205.

Roberts P.K. & Bilkei G. 2005. Field experiences on post-cervical artificial insemination in the sow. Reproduction in Domestic Animals, 40: 489-491.

Ron M., Blanc Y., Band M., Ezra E & Weller J.I. 1995. Misidentification rate in the Israeli dairy cattle population and its implication for genetic improvement. Journal of Dairy Science, 79: 676-681.

Rozeboom K.J., Reickes D.L. & Wilson M. 2004. The reproductive performance and factors affecting on-farm application of low-dose intrauterine deposit of semen in sows. Journal of Animal Science, 82: 2164 – 2168.

Serret C.G., Alvarenga M.V.F., Cória A.L.P., Dias C.P., Corcini C.D., Corrêa M.N., Deschamps J.C., Bianchi I. & Lucia T.Jr. 2005. Intrauterine artificial insemination in female swine with distinct sperm concentrations, parities and methods of ovulation estimation. Animal Reproduction, 2: 250-256.

Stahlberg R., Harlizius B., Weitze K.F. & Waberski D. 2000. Identification of embryo paternity using polymorphic DNA markers to assess fertilizing capacity of spermatozoa after heterospermic insemination in boars. Theriogenology, 53: 1365-1373.

Statistix® 8.0. Analytical software, user's manual. Tallahassee, FL, 2003. 396p.

Watson P.F. & Behan J.R. 2002. Intrauterine insemination of sows with reduced sperm numbers: results of a commercially based field trial. Theriogenology, 57: 1683-1693.

Weitze K.F., Wagner-Rietschel H., Waberski D., Richter L. & Krieter J. 1994. The onset of heat after weaning, heat duration, and ovulation as major factors in AI timing in sows. Reproduction in Domestic Animals, 29: 433-443.

Xu X., Pommier S., Arbov T., Hutchings B., Sotto W. & Foxcroft G.R. 1998. In vitro maturation and fertilization techniques for assessment of semen quality and boar fertility. Journal of Animal Science, 76: 3079–3089.

Xu X., Peng M., Fang Z. & Xu X. 2000. The direction of microsatellite mutations is dependent upon allele length. Nature Genetics, 24: 396-399.

Xue J.L., Dial G.D., Trigg T., Davies P. & King V.L. 1998a. Influence of mating frequency on sow reproductive performance. Journal of Animal Science, 76: 2962-2966.

Xue J.L., Lucia T., Koketsu Y., Dial G.D. & Marsh W.E. 1998b. Influence of mating frequency and weaning-to mating interval on sow reproductive performance. Swine Health and Production, 6: 157-162.