A Study on the Effect of *Pseudomonas aeruginosa* in Semen on Bovine Fertility

Matthew D. Eaglesome, Manuel M. Garcia and Andrzej B. Bielanski

ABSTRACT

Two experiments were done to demonstrate whether the presence of *Pseudomonas aeruginosa* in bovine semen could affect fertilization and/or early embryonic development. In the first experiment, superovulated heifers were inseminated with semen naturally contaminated with *P. aeruginosa (ADRI 102)* or clean semen and seven day-old embryos were collected nonsurgically. The endometrium of treated heifers appeared more sensitive to the flush procedures. In experiment 2, heifers were inseminated at synchronized estrus with semen experimentally contaminated with *P. aeruginosa (ADRI 102)* and processed in the same way as commercial semen with antibiotics (gentamicin, lincomycin, spectinomycin and tylosin) or processed without antibiotics added. Embryos were recovered at slaughter seven days later. In general, there was no significant reduction in fertility or development of embryos *in vitro* as a result of relatively high numbers of *P. aeruginosa* in bovine semen.

RÉSUMÉ

Deux expériences furent conduites afin de démontrer si la présence de *Pseudomonas aeruginosa* dans la semence bovine pouvait affecter la fertilisation et/ou le développement embryonnaire. Dans la première expérience, des taures étaient superovulées et inséminées avec de la semence contaminée naturellement avec *P. aeruginosa (ADRI 102)* ou de la semence non-contaminée avec ce micro-organisme et les embryons étaient récoltés non-chirurgicalement à l'âge de sept jours. L'endomètre des taures inséminées avec la semence contaminée a semblé plus sensible aux manipulations associées aux lavages de l'utérus pour obtenir les embryons. Dans la deuxième expérience, les taures étaient inséminées, au cours d'un oestrus synchronisé, avec de la semence contaminée expérimentalement avec *P. aeruginosa (ADRI 102)* et préparée de la même manière que la semence commerciale, avec des antibiotiques (gentamicine, lincomycine, spectinomycine et tylosin) ou préparée sans l'addition d'antibiotiques. Les embryons étaient obtenus sept jours plus tard, après abattage des animaux. En général, il n'y a pas eu de réduction de la fertilité ou du développement embryonnaire *in vitro* à la suite de la présence d'un nombre relativement élevé de *P. aeruginosa* dans la semence bovine. (*Traduit par Dr Patrick Guay*)

Pseudomonas aeruginosa occurs widely in nature and has a role as a commensal or contaminant in or on the body of healthy animals. However, it is an opportunistic pathogen and can cause disease when resistance to infection is low (1). The organism is frequently found in the prepuce of the bull and in semen. It can cause serious genital disease in artificial insemination (AI) bulls (2) and impair the functional ability of spermatozoa *in vitro* (3) but there are conflicting results as to whether it can cause poor conception and genital disease and abortion in female cattle when present in bull semen (4,5,6).

While its role in bovine genital disease is unclear, the AI industry takes steps to prevent transmission of the organism in semen by using hygienic measures in collecting and processing semen and by treating semen with antibiotics.

The objective of this study was to determine the effect of the organism on fertilization and/or early embryonic development of inseminated female cattle. The *P. aeruginosa* strain *ADRI 102* used in this study had been recently isolated from bull semen diluted and frozen for use in commercial AI (see Experiment 1). It appeared typical of strains commonly shed in bull semen.

The first experiment involved the evaluation of fertility and embryonic development using semen naturally contaminated with *P. aeruginosa*. Semen samples from three ejaculates of one bull (A) and one ejaculate from another bull (B) were used. The semen had been diluted with whole milk containing 1000 IU penicillin, 1,000 μg streptomycin, 300 μg lincomycin, 600 μg spectinomycin and 500 μg minocycline/mL diluted semen and frozen in straws for use in commercial AI. All three ejaculates from bull A were found to be contaminated with *P. aeruginosa*; heavy growth (>50 colonies to confluent growth per plate) from two ejaculates, and light growth (1–5 colonies per plate) from the third ejaculate. By contrast, semen from bull B was *Pseudomonas*-free. These results were based on three separate tests, one at the time of insemination. Fifteen heifers were selected based on normal estrous cycles and the absence of *P. aeruginosa* in cervical mucus. They were superovulated and inseminated at synchronized estrus and

Department of Pathobiology (Eaglesome, Bielanski) and Department of Microbiology (Garcia), Agriculture Canada, Animal Diseases Research Institute, NEPEAN, P.O. Box 11300, Station H, Nepean, Ontario K2H 8P9.

Submitted March 29, 1994.

TABLE I. Effect of *Pseudomonas aeruginosa*-free or -contaminated semen on fertility rate and development of embryos *in vitro* of superovulated heifers

Semen		Heifers			Embryos				
					0 h		24 h		
Bull	*Pseudomonas* contamination	Group	With embryos	Fertilization Rate (%)	Good	Poor	Good	Poor	Development[f]
A	heavy[a]	I	4	89	20	11	21	10	61
A	light[b]	II	4	79	13	6	14	5	74
B	none[c]	III	5	100	18	5	19	4	83

[a]Confluent growth
[b]1–5 colonies
[c]No growth of *Pseudomonas aeruginosa* per plate
[f]Percentage of embryos which developed further *in vitro* over 24 h
Percentages within a column are not different (p>0.05, Fischer Exact Test)

again 18 h later. They were divided into three groups and bred with the following: Group I — six heifers inseminated with semen heavily contaminated with *P. aeruginosa*; Group II — four heifers inseminated with semen lightly contaminated with *P. aeruginosa*; Group III — five heifers inseminated with *Pseudomonas*-free semen.

Embryos were recovered nonsurgically by flushing reproductive tracts seven days after estrus. Ova were assessed morphologically for fertilization and embryos for quality and for progress in development (7) at recovery and after 24 h *in vitro* culture in Ham's F10 medium (Gibco, Grand Island, New York) supplemented with 10% fetal calf serum (Gibco). After culture, the embryos were washed 3x in phosphate buffered saline pH 7.2 (PBS) and sonicated for 20 s to release any *P. aeruginosa* into the suspension. Droplets of thawed semen from each insemination straw, fluids from flushed uteri, embryo culture fluids, embryo wash fluids, and sonicated embryos were cultured for bacteria by plating on TSAB and incubation at 35°C for 18 h.

All samples of uterine flush fluids, embryo culture fluids and wash fluids and sonicated embryos from heifers of all three groups were negative for *P. aeruginosa* culture. However, dried pus materials were observed in the flush fluid of one group I heifer. These particulate materials were found to contain *P. aeruginosa*. All samples from group III heifers and from two heifers of group II were free of bacterial contamination. However, samples from the other two heifers from group II and all group I heifers showed non-*Pseudomonas* bacterial contamination (gram-positive and gram-negative bacteria, not identified) with more profuse contamination from the latter. Flush fluids from all group II and III heifers were clear and free of blood. By contrast, fresh blood was present in large amounts in fluids recovered from the uteri of all four of the group I heifers flushed, suggesting increased endometrial vascularity.

Fertility rates and embryo descriptions and development *in vitro* are recorded in Table I. The fertilization rates of *Pseudomonas*-treated heifers while somewhat lower than the control group, fell within the normal range of fertilization rates for superovulated heifers (8,9). Likewise, the quality and *in vitro* development rate of embryos from *P. aeruginosa*-treated heifers were lower than the control group.

For the second experiment, fresh cultures of *P. aeruginosa* were transferred from trypticase soy agar supplemented with 5% sheep blood (TSAB) (Difco Laboratories, Detroit, Michigan) to trypticase soy broth and incubated at 35°C for 18 h. The organisms were pelleted by low-speed centrifugation of broth culture at 1600 *g* for 20 min and resuspended in PBS. Direct microscopic counts were done immediately of a 1:10 dilution series to calculate the inoculum size. Plate counts on TSAB plates were also done as retrospective checks of the direct counts.

Fresh raw bull semen was brought to the laboratory and processed as described in a companion paper (10). Briefly, *P. aeruginosa* was added to the fresh semen to give an approximate concentration of 1×10^6 bacterial cells/mL, mixed and held for 15 min. Antibiotics (0.02 mL) were then added to the semen at concentrations of 500 μg gentamicin, 300 μg lincomycin, 600 μg spectinomycin and 100 μg tylosin (GLST)/mL of *Pseudomonas*-treated semen. Five minutes after mixing, the GLST treated samples were diluted 1:9 with homogenized whole milk or egg yolk Tris (EYT) containing GLST at the same concentration as the semen sample (11). The samples were incubated at 35°C for 20 min, cooled to 5°C (1°C/min), and 90 min later an equal volume of chilled milk or EYT containing 17% glycerol, but no antibiotics, was gradually added. The diluted semen was then frozen in 0.5 mL straws and stored in liquid nitrogen. *Pseudomonas*-treated semen was also processed and frozen exactly as described except for the absence of antibiotics.

Nineteen heifers were selected based on normal estrous cycles and the absence of *P. aeruginosa* in cervical mucus. They were not superovulated but were inseminated at synchronized estrus and again 18 h later; divided into four groups they were bred with the following: Group IV — five heifers inseminated with milk-diluted semen without antibiotics; Group V — five heifers inseminated with milk-diluted semen with antibiotics; Group VI — five heifers inseminated with EYT-diluted semen without antibiotics; Group VII — four heifers inseminated with EYT-diluted semen with antibiotics.

Embryos were recovered at slaughter by flushing reproductive tracts seven days after estrus. Assessment for fertilization and embryonic development was the same as for experiment 1.

Droplets of thawed semen from each insemination straw, fluids from flushed uteri, embryo culture fluids, embryo wash fluids, and sonicated embryos were cultured as in experiment 1.

Heavy growth of *P. aeruginosa* was recovered (confluent growth per plate) from all thawed semen samples diluted

Table II. Effect of *Pseudomonas aeruginosa* contaminated semen, with or without antibiotics added, on fertility rate of heifers and *in vitro* development of embryos

Semen			Heifers			Embryos				
						0 h		24 h		
Diluent	Antibiotics	Pseudomonas contamination	Group	Ovulated	Fertilization rate(%)	Good	Poor	Good	Poor	Development[1]
Milk	−	Heavy[a]	IV	4	100	3	1	3	1	0
Milk	+	Light[b]	V	5	80	3	1	2	2	25
EYT	−	Heavy	VI	3	100	3	0	3	0	100
EYT	+	None[c]	VII	4	50	0	2	0	2	0

EYT = Egg yolk Tris
+,- = Antibiotic-treated or nontreated semen
[a]Confluent growth
[b]1–5 colonies
[c]no growth of *Pseudomonas aeruginosa* per plate
[1]Percentage of embryos which developed further *in vitro* over 24 h

with milk or EYT but without antibiotics (Table II). All samples of antibiotic-treated semen diluted with milk were also contaminated with *P. aeruginosa* but culture growth was lighter (1–5 colonies per plate). However, antibiotic-treated semen samples diluted with EYT were *Pseudomonas*-free.

All samples of uterine flush fluids, embryo culture fluids and wash fluids, and sonicated embryos from heifers of all four groups were negative for *P. aeruginosa* culture. All samples from group V and VII heifers, except for two culture media samples from two heifers of group V, were free of other bacterial contamination. However, culture of sample from all group IV and group VI heifers showed discrete colonies of both gram-positive and gram-negative bacterial contaminants; growth was light to moderate but insufficient to hinder detection of any *P. aeruginosa*. Another species *Xanthomonas*, (formerly *P. maltophilia*) was cultured from all samples from one of the Group VI heifers. Fertility rates and embryo descriptions and development *in vitro* are recorded in Table II. Breeding heifers with milk or EYT-diluted semen heavily contaminated with *P. aeruginosa* had no detrimental effect on fertilization or embryo quality, nor using EYT-diluted semen on embryo development *in vitro* (Table II). However, bacterial contamination levels were high in uterine fluids, embryo culture and wash fluids, and embryos recovered from heifers bred with *Pseudomonas*-contaminated semen.

To control bacterial contamination, antibiotics such as the two combinations used in this study are added to bull semen processed for AI. Neither combination consistently eliminated *P. aeruginosa* from experimentally or naturally contaminated semen, confirming a previous report (10). The question then arises as to whether the survival of the organism in antibiotic-treated semen should be of concern to animal breeders. Our observation was that there was no apparent effect on fertilization, embryo quality or development *in vitro* attributable to *P. aeruginosa* strain *ADRI 102* isolated from bull semen. While there was a higher level of non-*Pseudomonas* bacterial invasion in treated heifers and an apparent increase in endometrial vascularity in superovulated and treated heifers, *P. aeruginosa* was subsequently recovered from only one of the treated heifers. Follow-up studies need to be done to determine whether specific virulence factors in *P. aeruginosa* may influence bovine fertility.

ACKNOWLEDGMENTS

We wish to thank Eastern Breeders Inc., Kemptville, Ontario and United Breeders, Guelph, Ontario for kindly supplying the bull semen.

REFERENCES

1. **BERGAN T.** Human- and animal-pathogenic members of the genus *Pseudomonas*. In: Starr MP, Stolp H, Truper HG, Balows A, Schlegel HG, eds. The Prokaryotes. New York: Springer-Verlag, 1981: 666–700.
2. **CORRIAS A, MOLINARI G.** Serious genital infection due to *Pseudomonas aeruginosa* in artificial insemination bulls. Atti Soc Ital Sci Vet 1958; 12: 243–248.
3. **BORYCZKO VW.** Influence of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* types on metabolic processes and vitality of spermatozoa. Zuchthyg 1982; 17: 13–18.
4. **GUNSALUS JC, SALISBURY GW, WILLETT EL.** The bacteriology of bull semen. J Dairy Sci 1941; 24: 911–919.
5. **CORRADINI L, BINATO L.** Su un caso di aborto da *Pseudomonas* nel bovino. Veterinaria Ital 1961; 12: 320–322.
6. **WIERZBOWSKI S.** Bull semen opportunistic pathogen and ubiquitary microflora. In: FAO, Disease Control in Semen and Embryos. FAO Animal and Health Paper No. 23. 1981 (Reprinted 1985): 21–28.
7. **LIDNER KJ, WRIGHT WR Jr.** Bovine embryo morphology and evaluation. Theriogenology 1983; 20: 407–416.
8. **DONALDSON LE.** Embryo production in superovulated cows: transferable embryos correlated with total embryos. Theriogenology 1984; 21: 517–524.
9. **BIELANSKI A, YADAV BR.** A note on fertilization and embryo production in superovulated cattle with various levels of subcutaneous fat tissue. Anim Prod 1990; 51: 426–430.
10. **EAGLESOME MD, GARCIA MM.** Comparisons of antibiotic combinations to control *Pseudomonas aeruginosa* in bovine semen. Can J Vet Res 1995; 59: 73–75.
11. **SHIN SJ, LEIN DH, PATTEN VH, RUHNKE HL.** A new antibiotic combination for frozen bovine semen. 1. Control of mycoplasmas, ureaplasmas, *Campylobacter fetus* subsp. *venerealis* and *Haemophilus somnus*. Theriogenology 1988; 29: 577–591.