Evaluation of the embryo transfer procedure proposed by the International Embryo Transfer Society as a method of controlling vertical transmission of *Neospora caninum* in cattle

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Objective—To evaluate efficacy of embryo transfer into seronegative recipients, using the procedure proposed by the International Embryo Transfer Society (IETS), for preventing vertical transmission of *Neospora caninum* in cattle.

Design—Prospective clinical trial.

Animals—87 recipient cows and heifers and their embryo transfer calves from 22 donors originating from 9 dairy herds.

Procedure—*Neospora caninum* serologic status of donors and recipients was determined before collection and transfer of embryos. Viable embryos were washed and treated with trypsin. Recipients in experimental groups A (n = 50) and B (29) were seronegative and received embryos from seropositive and seronegative donors, respectively. Recipients in group C (n = 8) were seropositive and received embryos from seronegative or seropositive donors. Antibody titers against *N caninum* were determined monthly during pregnancy in recipients and in calf blood samples collected at birth. Tissues collected from stillborn calves and aborted fetuses were analyzed histologically and by immunohistochemical (IHC) methods.

Results—76 calves and 11 fetuses and stillborn calves were examined. All calves from groups A and B were seronegative (n = 70) or lacked evidence of infection by use of tissue analysis (9). In group C, 5 of 6 calves were seropositive at birth, and IHC results were positive for 1 of 2 calves. Vertical transmission rate was significantly lower in groups A and B (0%) than in group C (75%).

Conclusion and Clinical Relevance—Embryo transfer into seronegative recipients, using the procedure proposed by IETS, is an effective way to prevent vertical transmission of *N caninum*. Results provide support for pretransfer testing of all embryo transfer recipients. (*J Am Vet Med Assoc* 2001;218: 1803–1806)

Neospora caninum was identified for the first time in 1988 in dogs.[1] The first abortion associated with *Neospora* spp in cattle was reported in a New Mexico herd in 1988.[2] Numerous reports across the world have since indicated that *N caninum* is an important cause of abortion in cattle.[3]

Although horizontal transmission through a definitive host likely occurs,[4-6] vertical transmission through in utero infection is the most common mode of transmission.[4,7-9] Serologic data and results of genealogic analyses support this observation.[4,7,10-13] Estimated rate of vertical transmission varies from 44.4 to 100% among studies.[4,14-17] Vertical transmission alone can maintain infection through generations in a herd.[14]

Based on present knowledge, the goals of a control strategy for *N caninum* are to prevent vertical transmission of the parasite[18,a] and minimize risk of horizontal transmission.[16] Culling of seropositive cattle has been proposed[a] and studied with mathematical models to determine efficacy.[19] However, for some herds this results in the loss of valuable animals with superior genetic merit and high commercial value.

Protecting the early-stage embryo from exposure to viral or bacterial contamination decreases the risk of disease transmission to the embryo and recipients.[20,21] For transmission of an infectious agent to occur, it must be present in the embryonic cells, associated with the zona pellucida, or present in the medium used to transfer the embryo.[22,23] Embryo transfer, after washing and trypsin treatment of the embryos as recommended by the **International Embryo Transfer Society (IETS)**, is a proven method for effectively controlling transmission of pathogens such as bovine leukosis virus, bovine viral diarrhea virus, infectious bovine rhinotracheitis virus, and *Brucella abortus*.[23] This procedure prevents disease transmission by use of visual inspection for integrity of the zona pellucida before and after the procedure and by removal of debris and foreign materials surrounding the embryos during the 10 serial passages and gentle shaking into the washing medium. Each washing passage represents a 1:100 dilution of the medium surrounding the embryo. Trypsin treatment of embryos that have an intact zona pellucida effectively removes or inactivates certain viruses.[23]

The objective of the study reported here was to determine the efficacy of embryo transfer into seronegative recipients, using the procedure defined by the IETS, to prevent vertical transmission of *N caninum* in cattle.

Material and Methods

Selection of cattle—Eleven Holstein herds from southwestern Québec were selected to participate in this project.

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Participating herds had a history of abortions confirmed to be caused by *N caninum* or involving seropositive cattle, determined on the basis of Clinique Vétérinaire St-Louis health records. Precedence was given to herds with computerized health records. Availability of sufficient number of potential donors and recipients in order to constitute defined experimental groups before July 1999 was also considered a selection criterion. Each herd included in the study was required to provide 1 seronegative and 1 seropositive donor and at least 12 recipients of which 2 were seropositive.

Experimental groups—Group A, the experimental group, comprised 50 seronegative recipients that received embryos collected from seropositive donors. The objective of this group was to test the hypothesis that embryo transfer into seronegative recipients effectively prevents vertical transmission of *N caninum*. Group B, the control group, comprised 29 seronegative recipients that received embryos collected from seronegative donors. Group C comprised 8 seropositive recipients implanted with embryos collected from either seronegative or seropositive donors. Group C was used to confirm transmission of the parasite from an infected recipient to her offspring. Participant breeders received compensation for females confirmed to be infected at birth in group C.

Embryo transfer protocol—Sample size for embryo donors (n = 22) was chosen on the basis of estimated values[b] for superovulation rate (90%), mean number of viable ovulations per collection (5.75), mean pregnancy rate in recipients (60%), and mean embryo loss rate in recipients (5%), which suggested that 66 successful pregnancies would result. Superovulatory treatment was initiated either between days 9 and 14 of the estrus cycle when previous estrus was observed and recorded or 96 hours after administration of 3 mg of estradiol 17β[c] preceded by insertion of a progesterone-releasing device[d] 24 hours earlier. Gonadotropin treatment of donors consisted of 400 mg of follicle-stimulating hormone[e] administered twice daily IM in a decreasing dosage schedule for 5 days. Luteolysis was induced by administration of 40 mg of dinoprost[f] 72 hours after the first gonadotropin treatment. Cattle were artificially inseminated twice, 54 and 72 hours after dinoprost administration.

Nonsurgical embryo collection was performed 6.5 to 7 days after estrus. Embryos were evaluated, categorized, washed, and treated with trypsin according to standards set by the IETS.[23,24] Embryos of stage 4 to 7 and of quality 1 and 2 were defined as viable.[24] Visual inspection of embryos to ensure integrity of the zona pellucida was performed before and after the washing and trypsin treatment procedure. This procedure was performed by transferring embryos through 5 Petri dishes containing a complex serum-free solution,[g] then through 2 dishes containing 0.25% trypsin in Hanks balanced salt solution,[h] and finally through 5 dishes of the same medium as in the first 5 washes.[24]

Embryos were transferred nonsurgically into synchronized recipients or frozen in 1.5*M* ethylene glycol plus 0.1*M* sucrose, using a standard procedure.[25] Frozen embryos were thawed and transferred via a described procedure.[25] After reviewing available data on serologic and synchronization status of recipients, the **corpus luteum (CL)** was evaluated and localized, and epidural anesthesia was performed. Frozen embryos were thawed at 30 C (86 F) in a water bath for 30 seconds and transferred into the cranial third of the uterine horn ipsilateral to the CL.

Recipients were examined by use of transrectal ultrasonography 27 to 33 days after transfer for the presence of a conceptus, and its viability was assessed by observation of a heartbeat. Pregnancies were reconfirmed by use of transrectal palpation after 60 days. Blood was collected monthly from pregnant recipients, and sera were stored at –20 C (–4 F) for up to 30 days and then at –70 C (–94 F) for permanent storage until submission to the laboratory. All samples were tested simultaneously at the end of the study.

Aborted fetuses and stillborn calves were necropsied, and brain, heart, diaphragm, skeletal muscle, lung, liver, and kidney were collected. Blood samples were collected from calves at birth, before colostrum intake, to determine precolostral *N caninum* antibody titer. Udder supports were provided to participant herds to avoid accidental colostrum intake. Serum activity of **λ-glutamyltransferase (GGT)** was measured for each calf to confirm that the sample was precolostral. Serum GGT values < 50 U/L were interpreted as indicating that the serum sample was obtained before ingestion of colostrum.[26]

Laboratory procedures—Serologic testing was performed by use of an ELISA with a reported sensitivity of 88.4% and specificity of 99.0% at a cutoff ratio of 0.60.[i] Results were expressed as a ratio between **optical density (OD)** of the sample and OD of a positive standard. To maximize sensitivity and specificity, a suspect zone between ratios of 0.41 and 0.79 was considered. Recipients were considered seronegative, retrospectively, if initial ELISA ratio was < 0.41 and did not exceed 0.79 more than once during pregnancy. Recipients were considered seropositive, retrospectively, if initial ELISA ratio was > 0.79 and did not decrease below 0.41 more than once during pregnancy. These criteria allowed for misidentification of samples. Histologic examination and **immunohistochemical (IHC)** analysis were performed on the brain and heart of aborted and stillborn calves by use of a standard procedure.[27,j]

Statistical analysis—Rate of vertical transmission of *N caninum* was obtained by dividing the number of infected calves or fetuses by the number of calves and fetuses tested in each group. A Fisher exact test was used to compare the rate of vertical transmission among the 3 study groups. A 95% **confidence interval (CI)** was calculated for each estimated transmission rate, assuming a normal distribution. Mann-Whitney and Fisher exact tests were used to compare results of embryo transfer and fetal and embryonic losses between seropositive and seronegative recipients. An α value of 0.05 was used for all statistical analyses.

Results

Screening—Four hundred thirty-seven heifers and cows from 11 different herds were serologically tested for *N caninum*. Two herds could not provide the minimal number of potential donors and recipients and were excluded from the study. Of all cattle tested, 72 (16.5%) were rejected, because their ELISA ratio was between 0.41 and 0.79. Twenty-nine (7%) had an ELISA ratio > 0.79 and were retained as potential seropositive donors or recipients. Three hundred sixteen (76%) had an ELISA ratio < 0.41 and were retained as potential seronegative donors or recipients. Overall seroprevalence was 9.8% (43/437) at a cutoff ratio of 0.6 used for a previous seroepidemiologic investigation.[16]

Superovulation and embryo transfer—Twenty-two superovulations and embryo collections were performed in 9 different herds on 11 seropositive and 11 seronegative donors. Two hundred nine embryos were collected for an overall mean of 9.5 viable embryos/donor, including the 4 donors (1 seropositive and 3 seronegative) that did not produce any viable embryos. Seropositive and seronegative donors produced 126 (11.5/donor) and 83 (7.5/donor) viable embryos, respectively ($P = 0.25$).

Of the 209 embryos collected, 174 were transferred to constitute the study groups (group A, 101 embryos [30 fresh]; group B, 57 embryos [22 fresh]; group C, 16 embryos [9 fresh]). In group C, the 16 seropositive recipients received

embryos collected from 8 seronegative and 8 seropositive donors. One hundred nine (63%) pregnancies were confirmed (group A, 63 [62%]; group B, 36 [63%]; group C, 10 [63%]). Pregnancy rate in group A for cattle that received fresh or frozen embryos was 67 and 61%, respectively. Pregnancy rate in group B cattle that received fresh or frozen embryos was 68 and 60%, respectively. Pregnancy rate in group C cattle that received fresh or frozen embryos was 78 and 43%, respectively. Embryo losses or abortions occurred in 4 (6%) cattle in group A, 4 (11%) in group B, and 3 (30%) in group C ($P = 0.07$).

Overall, 87 gestations yielded study results (group A, 50; group B, 29; group C, 8, with 5 from seropositive donors). Seven confirmed pregnancies did not come to term before the end of the study, and for 11 pregnancies, blood and tissues were not collected at birth. Four pregnancies did not meet inclusion criteria determined for the study: 2 recipients in group A had ELISA ratios > 0.79 for 2 and 3 tests performed during pregnancy, another recipient in group A was omitted from 1 monthly testing during pregnancy and had 1 ELISA ratio > 0.79, and 1 recipient in group C had 4 ELISA ratios < 0.41 during pregnancy. Misidentification of the pretransfer sample was suspected in 3 of these recipients.

Serologic examinations were performed on 43 calves in group A, 27 in group B, and 6 in group C. All calves in groups A and B and 1 calf in group C were seronegative at birth. One seronegative calf with increased serum activity of GGT (6,869 U/ml) in group B was included in calculations of vertical transmission. In group C, 5 calves were seropositive at birth (ELISA ratio > 1.16). Histologic and IHC examinations were performed on 7 calves in group A, 2 in group B, and 2 in group C. Results of histologic and IHC examinations were positive on tissues of 1 aborted fetus in group C and negative on tissues of the remaining 10 calves and fetuses. On the basis of serologic or tissue analysis, all calves in groups A and B were considered free of infection. Six of the 8 calves in group C were considered infected at birth. Two calves in group C, 1 from a seropositive donor and 1 from a seronegative donor, were considered free of infection.

For groups A, B, and C, vertical transmission rates were 0 of 50 (0%; CI, 0 to 5.8%), 0 of 29 (0%; CI, 0 to 9.8%), and 6 of 8 (75%; CI, 35.6 to 95.5%), respectively. The lower limit of the CI for group C was beyond the upper limits of the CI for groups A and B, and differences between group C and groups A and B were significant ($P < 0.001$) .

Discussion

The embryo transfer procedure proposed by the IETS includes transfer of embryos into seronegative recipients and is an effective method of producing calves that are free of infection with *N caninum* at birth. All calves born from seronegative recipients (groups A and B) had negative results of serologic tests or tissue analysis at birth and, therefore, were considered free of infection.

The high rates of vertical transmission observed in seropositive recipients (group C) and the absence of vertical transmission in seronegative recipients (groups A and B) confirm that the risk of new infection is linked to the serologic status of the recipients. The finding that seronegative recipients gave birth to seronegative calves after receiving embryos from seropositive donors supports the conclusion that the embryo transfer procedure as recommended by the IETS removes the potential risk represented by the serologic status of the donor. Therefore, it appears logical and justifiable to select potential recipients that are seronegative. Because our study did not use untreated embryos, it is presently unknown whether or not the IETS procedure is essential to prevent transmission of *N caninum*.

Seroprevalence in purchased cattle has been reported to exceed prevalence among cattle raised and maintained on farms.[28] Seroprevalence in participating herds (9.8%) was similar to that of other reports.[16] We hypothesize that seroprevalence in the population of potential embryo transfer recipients available in the market place may be higher than the reported seroprevalence for that area (16.6%).[16] This high seroprevalence could be a drawback of culling seropositive cattle as a control measure for neosporosis in dairy herds. Breeders relying on purchased recipients without screening are at risk of increasing prevalence of infection by *N caninum* in their replacement herd. Intensive use of embryo transfer without pretesting recipients of unknown origin may represent a substantial biosecurity risk for introduction of *N caninum* into a herd and contamination of valuable uninfected cattle.

In a review[29] of different serologic tests, it was concluded that proper categorization of animals by use of a single test remains a challenge in field conditions. At a cutoff ratio of 0.41, the ELISA used in the study reported here was an effective way of selecting noninfected females for use as embryo transfer recipients. A substantial portion (16.5%) of females sampled in participating herds had a ELISA ratio between 0.41 and 0.79. Risk of vertical transmission of *N caninum* in cattle such as these is unknown and will require further studies.

Blood samples collected from calves were assayed for serum activity of GGT to confirm that calves had not received colostrum. For 1 calf, serum activity of GGT was > 50 U/ml; this calf was included in study results because of its negative serologic status. Colostrum intake could not have decreased its antibody titer.

Serologic status did not appear to influence the donor's potential for embryo production. The number of embryos collected and pregnancy rate were not statistically different between the 2 groups of donors. Mean number of viable embryos per donor was greater than typically reported.[b] Parity and reproductive status of donor may have positively influenced superovulation results. Mean parity of seropositive donors was 2.8 lactations, whereas parity of seronegative donors was 3.5 lactations. Only 1 seropositive donor was a repeat breeder at time of superovulation.

Reported risk for pregnancy loss is about 3 times greater for seropositive females, compared with seronegative herdmates,[15,30,31] and in some situations is as much as 7 times greater.[31,32] Stillbirth rates have reportedly increased by a factor of 2.5.[32] Rate of pregnancy loss observed in groups A and B appeared lower than in group C, but sample size in the latter group likely precluded identification of a significant difference. On the basis of this observation and available data in the literature, serologic screening for *Neospora* status of potential embryo transfer recipients could be immediately rewarding by decreasing the rate of pregnancy losses.

Despite the anecdotal reports on this subject, this

is, to the authors' knowledge, the first scientific report to confirm that embryo transfer, using a procedure proposed by the IETS, into seronegative recipients is an effective way of preventing vertical transmission of *N caninum* in cattle. This will benefit breeders by decreasing the reproduction and production losses related to *N caninum* in their herds and allowing for development and marketing of noninfected bloodlines.

[a]Paré J. Epidemiology of *Neospora caninum* in cattle, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, 1996.
[b]Mapletoft R, University of Saskatchewan, Saskatoon, SK, Canada: Personal communication, 1998.
[c]Courtesy of Giroux D, St-Hyacinthe, QC, Canada.
[d]CIDR, Vetrepharm Canada Inc, London, ON, Canada.
[e]Folltropin, Vetrepharm Canada Inc, London, ON, Canada.
[f]Lutalyse, The Upjohn Company-Animal Health Division, Orangeville, ON, Canada.
[g]Vigro Holding Plus, ABTechnology, Pullman, Wash.
[h]Life Technologies, Burlington, ON, Canada.
[i]Biovet, St-Hyacinthe, QC, Canada.
[j]Prairie Diagnostic Services, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada.

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