RISK OF A BRUCELLA TRANSMISSION BY PORCINE EMBRYOS: AN IN VITRO STUDY

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Introduction
The cryoconservation of pig embryos with intact zona pellucida would be a major advantage to control and then insure minimal risk of disease transmission during embryo transfer of genetic material. Moreover, international rules on embryo transport outside of the country origin require that only embryos with an intact zona pellucida may be exported [9]. A vitrification technique is currently being studied in several species to resolve the problems of cryopreservation. Recently, a new technique has been developed to increase the cooling and warming rates of vitrification named Open Pulled Straw (OPS) technology [11]. The faster cooling rate seems to be one of the key solutions to protect porcine embryos from chilling injuries and to obtain piglet births after vitrification of unhatched blastocysts [2,3]. The OPS method allows the conservation of genetic resources and opens possibilities for porcine embryo transfer industry. In particular, exchange of genetic material between countries may then be simplified.

However, pathogens could be cotransferred with embryos. Transmission by embryo transfer of viruses to swine has been studied [10], and transmission of bacteria has been few investigated [7]. This study reports the evaluation of risks of transferring Brucella species via porcine embryos. The choice of Brucella genus relies on the re-emergence of brucellosis in pigs due to Brucella suis biovar 2 responsible for abortion, orchitis and sterility in swine, since 1993, in France and on the enzootic presence of this biovar in other European countries [5].

Material and Methods
1- Embryo production and collection
Superovulated Large White hyperprolific gilts (n=20) were used as embryo donors. Gilts were artificially inseminated 12 and 24 h after initial detection of oestrus using fresh semen. On days 5.5 or 6 of the oestrous cycle (Day 0= onset of oestrus) they were slaughtered after electronarcosis, and their reproductive tracts were immediately removed. Embryos were recovered by flushing the uterine horns with phosphate buffer at 39°C containing 2% New Born Calf Serum. Embryo development stages were evaluated under a stereomicroscope, with 20x magnification. Only unhatched blastocysts (n=394) were selected and assigned for this study.

2- Embryos washings before contamination with Brucella species
Before contamination with Brucella, blastocysts were washed 10 times according to the recommendations of the International Embryo Transfer Society (IETS) for bovine embryos transfer. However, antibiotics have been used only in the 5 first washings-steps to avoid action on the further infection with Brucella strains.

3- Contamination with four Brucella strains
Blastocyst stage embryos from Large White hyperprolific gilts were selected (from 10 to 35 embryos per gilt). They were submitted in vitro, in 6-wells microplates (Costar, Corning, USA) at the rate of 1 to 8 embryos per well to a massive infection (10⁵ cfu/mL) with one of the four Brucella strains:
- Brucella abortus biovar 1 reference strain 544, isolated from bovine,
- Brucella suis biovar 2, and Brucella melitensis biovar 3, two wild strains isolated from swine, and
- Brucella ovis, a wild strain isolated from ram.
The Brucella strains and the embryos were incubated in the M199 culture media at 39°C with 5% CO₂. Brucella multiplication was measured after 17h of incubation, by culture of the M199 incubation media on trypticase soy agar yeast medium (TSA-YE) or TSA-YE added with 5 % horse serum for B. ovis (TSA-YES), after dilution in PBS. One hundred and seventy six embryos from ten sows were used in each trial.

Trial 1. After 17h of incubation with Brucella, embryos were washed 10 times with Dulbecco’s PBS, without antibiotics.

Trial 2. The second trial is incubated like the 1st one, except that washings have been performed with Dulbecco’s PBS added with penicillin (100IU/mL) and streptomycin (100µg/mL), as preconised by the IETS for bovine embryos transfer.

In both trials, viability of embryos after washings was controlled by observation under a stereomicroscope.

4- Washing bath controls
Washing baths number 1, 5, and 10 were cultured on TSA-YE or TSA-YES, to look for the presence of Brucella. Moreover, all washed embryos themselves were washed after the 10th washing and cultured also to look for the presence of Brucella.

Results
The control of Brucella culture from the M199 media showed that Brucella were still alive and were recovered on both TSA-YE or TSA-YES plates, after 17h of incubation in M199 media (data not shown). Enumeration showed that Brucella had survived or proliferated depending on the assays.

Stereomicroscopic observation of embryos after 17h of incubation with Brucella, did not show any alteration. In the 1st trial using Dulbecco’s PBS without antibiotics (Table 1A), Brucella strains were always massively recovered in the first washing samples. Only 10 to 30 % of the 5th washing samples were positive and in addition the cultures were then less intense. Brucella were never recovered in the last washing. Moreover, Brucella were detected from 10 to 30% of the washed embryos, according to the strains (the density of the culture was similar to that observed in the 5th washings).
Table 1. Brucella recovered in washing baths or embryos after 17h of incubation at 39°C, expressed in percentage of positive culture for each strain and each trial (the number of embryos tested are given between brackets).

<table>
<thead>
<tr>
<th>Washing bath</th>
<th>B. abortus biol. 1 (45)</th>
<th>B. melitensis biol. 3 (45)</th>
<th>B. suis biol. 2 (44)</th>
<th>B. ovis (42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>5</td>
<td>30</td>
<td>10</td>
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<td>0</td>
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<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Embryo</td>
<td>30</td>
<td>20</td>
<td>10</td>
<td>10</td>
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</tbody>
</table>

B. With antibiotics in the 10 washings

<table>
<thead>
<tr>
<th>Washing bath</th>
<th>B. abortus biol. 1 (44)</th>
<th>B. melitensis biol. 3 (43)</th>
<th>B. suis biol. 2 (44)</th>
<th>B. ovis (45)</th>
</tr>
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<tr>
<td>1</td>
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<td>5</td>
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<td>0</td>
</tr>
<tr>
<td>Embryo</td>
<td>0</td>
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In the 2nd trial (Table 1B) using antibiotics in the washing buffer, B. suis was never recovered whatever the washing step. Unlike B. suis, other strains were recovered in the 1st washing and only in 10% of the 5th washing for B. melitensis and B. abortus. No Brucella culture was positive for the 10th washing. Moreover, all the washed embryos were free of Brucella.

Discussion

Most of the studies concerning the sanitary risks of Brucella transmission by embryo transfer previously published were done on cows and showed that in vivo, artificially or naturally infected cows [1,4,8] did not seem to retain Brucella in their uterus. Transfer of cryopreserved cow embryos is now well documented whereas pig embryo transfers are more recent because of the sensitivity of these embryos to damage caused by cryopreservation. This problem being now solved by the development of the OPS method, we tried to document the critical point of the potential cotransfer of bacteria, and particularly Brucella, with embryos.

The inoculum of Brucella strains used in this study is extremely high. It was chosen to apply drastic conditions being probably over what is expected in practice. However, the growth of embryos is not disturbed after 17 hours of incubation with Brucella. Ten percent of them were hatched blastocysts at the end of culture. Results obtained could be a good indication that if in vitro, no Brucella are recovered, in vivo, Brucella would also probably be removed. These results evidenced the need of antibiotics. We have demonstrated that without antibiotics in the washings, if no Brucella were recovered from the 10th washing, embryos were still contaminated (from 10 to 30%). On the opposite, the trial using antibiotics as recommended by the IETS, showed no Brucella neither in the last washing nor on the embryos. Moreover, no B. suis were recovered whatever the washing sample cultured. Even if our Brucella inoculum is higher, our results are consistent with those obtained by Mallek et al. [6], on the effects of in vitro contamination by Brucella abortus (10⁴ to 10⁵ cfu/mL) on both mice and cows embryos who have demonstrated that ten washings were sufficient to eliminate Brucella from the transfer medium. However they did not use antibiotics in their media. Similar results were obtained with porcine embryos in vitro infected by various pathogens such as Pasteurella multocida or Streptococcus suis by Smits et al. [7], integrating a cocktail of penicillin and streptomycin in the washing bath, that corroborate ours.

Conclusion

These results emphasize the need of antibiotics in the buffer used to wash embryos before performing vitrification and transfer. Considering that the level of contamination with Brucella used in these essays is probably higher than what is expected in practice, we could conclude on the real effectiveness of the washings. According to this study, the application of washing procedures with antibiotics is necessary and sufficient to allow porcine embryos transfers without risk of transmission of Brucella.

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References


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