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 (10^6 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% CO_2. *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 mashed 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 17 h 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 mashed 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).

A. *Without antibiotics in the 10 washings*

Washing bath	*B. abortus* biov.1 (45)	*B. melitensis* biov. 3 (45)	*B. suis* biov. 2 (44)	*B.ovis* (42)
1	100	100	100	100
5	30	10	10	0
10	0	0	0	0
Embryo	30	20	10	10

B. *With antibiotics in the 10 washings*

Washingbath	*B. abortus* biov.1 (44)	*B. melitensis* biov. 3 (43)	*B. suis* biov. 2 (44)	*B. ovis* (45)
1	100	100	0	40
5	10	10	0	0
10	0	0	0	0
Embryo	0	0	0	0

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* (10^1 to 10^5 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 emphasise 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 assays 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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