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Transmission of *Mycoplasma bovis* infection in bovine *in vitro* embryo production

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ABSTRACT

Mycoplasma bovis (*M. bovis*) causes several costly diseases in cattle and has a negative effect on cattle welfare. There is no effective commercial vaccine, and antimicrobial resistance is common. Maintaining a closed herd is the best method to minimize the risk of introduction of *M. bovis*. Assisted reproduction is crucial in a closed herd to make genetic improvements. *M. bovis* has been found in commercial semen, and contaminated semen has been the source of disease in naïve dairy herds. The objective of this study was to evaluate *M. bovis* transmission in bovine *in vitro* embryo production (IVP) using several possible exposure routes. We used a wild-type *M. bovis* strain isolated from semen at a final concentration of 10^6 CFU/mL to infect cumulus–oocyte complexes, spermatozoa, and 5-day-old embryos. We also used naturally contaminated semen in fertilization. Blastocysts were collected on day 7–8 and zona pellucida (ZP)-intact embryos were either washed 12 times, including trypsin washes as recommended by the International Embryo Technology Society (IETS), or left unwashed. Washed and unwashed embryos, follicular fluids, maturation medium, cumulus cells, fertilization medium, and G1 and G2 culture media, as well as all wash media were analyzed using enrichment culture followed by real-time PCR detection of *M. bovis*. Altogether, 76 pools containing 363 unwashed embryos and 52 pools containing 261 IETS washed embryos were analyzed after oocytes, spermatozoa, or 5-day-old embryos were infected with *M. bovis* or naturally contaminated semen was used in fertilization. We could not detect *M. bovis* in any of the embryo pools. *M. bovis* was not found in any of 12 wash media from different exposure experiments. *M. bovis* did not affect the blastocyst rate, except when using experimentally infected semen. Contrary to an earlier study, which used a cell co-culture system, we could not demonstrate *M. bovis* in embryo wash media or tight adherence of *M. bovis* to ZP-intact embryos. Naturally infected semen did not transmit *M. bovis* to embryos. We conclude that by using our IVP system, the risk of *M. bovis* transmission via IVP embryos to recipient cows is very low.

1. Introduction

Mycoplasma bovis (*M. bovis*) causes a variety of clinical diseases in cattle, including mastitis, pneumonia, arthritis, otitis media, keratoconjunctivitis, and genital disorders [1,2]. Considered an emerging pathogen in the cattle industry worldwide, *M. bovis* recently emerged in Finland in 2012 [3] and in New Zealand in 2017 [https://www.mpi.govt.nz/protection-and-response/mycoplasma-bovis/]. The prevention of *M. bovis* infections remains difficult due to the lack of an effective commercial vaccine [4]. *M. bovis* infections have a debilitating effect on animal welfare, given their chronic nature and poor response to antimicrobial therapy [1]. Difficulties in determining the full economic cost of *M. bovis* to the cattle industry stem from the lack of accurate prevalence figures regarding clinical disease. However, costs obviously associate with reduced production, medicines and labor for treatment, death, and culling losses, as well as the financing of laboratory diagnostics and control measures. In dairy herds, the best practice for the prevention and

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control of *M. bovis* infections relies on maintaining a closed herd. The use of assisted reproduction technologies (ART), including artificial insemination (AI) and embryo transfer, remains crucial in closed dairy herds in order to ensure genetic improvements. We recently demonstrated that contaminated commercial AI sperm served as a source of *M. bovis* infection in closed naïve dairy herds [5], but only a few studies have examined the risk of *M. bovis* transmission via embryo transfer [6–8].

M. bovis infections in the female genital tract remain incompletely described [9,10]. Experimental infection studies and insemination with semen contaminated with *M. bovis* have resulted in salpingo-oophoritis and infertility [10]. Some studies have demonstrated that *M. bovis* infection leads to aborted fetuses [11,12]. In general, consensus holds that genital tract infections only occur in a few individual animals. Thus, the risk of *M. bovis* in collected ova might be low. However, the risk from using *M. bovis*-contaminated semen in *in vitro* fertilization might be moderate.

According to the Manual of the International Embryo Technology Society (IETS), *Mycoplasma* spp. Represents a category 4 pathogen in cattle. That is, "the risk of transmission via embryo transfer might not be negligible even if the embryos are properly handled in accordance with the IETS Manual between collection and transfer." This only applies to *in vivo*-derived embryos. Several studies have demonstrated that the bovine *zona pellucida* (ZP), the most important barrier against pathogens in an embryo, is thinner and more porous in *in vitro*-derived (IVP) embryos than in those that are *in vivo*-derived [13]. Therefore, the IETS manual states that the washing procedure might be inadequate for the removal of certain pathogens from *in vitro*-produced embryos. Indeed, Bielanski et al. [7] illustrated that IVP embryos produced using *M. bovis*-contaminated sperm during fertilization could not be rendered *M. bovis*-free using IETS-recommended washes. Previously, Bielanski et al. [6] found that 7-day-old *in vivo*-derived embryos exposed to *M. bovis* tested positive in an *M. bovis* culture despite IETS-recommended washes, including trypsin treatment.

Since 2016, the worldwide number of viable *in vitro*-produced embryos has exceeded the number of *in vivo*-derived transferable embryos [14]. Given this and evidence related to the close association of *M. bovis* with the bovine ZP, substantial economic costs and negative animal welfare effects may impact the cattle industry. Here, we examined the risk of *M. bovis* transmission in an *in vitro* embryo production system using both naturally and experimentally infected semen, as well as experimentally infected oocytes.

2. Materials and methods

2.1. IVP procedures

2.1.1. Bovine oocyte collection

Ovaries were collected from cows approximately 30 min after slaughter. The ovaries were rinsed in a sieve with ambient temperature physiological saline (B. Braun Melsungen AG, Melsungen, Germany) and then transferred to 1-L transport containers with physiological saline at ambient temperature. The containers were placed in a styrofoam box and immediately transported to the laboratory.

2.1.2. In vitro maturation

Cumulus–oocyte complexes (COCs) were aspirated from follicles of 2–8 mm in diameter within 6 h after slaughter using an 18-gauge needle and 10-mL syringe. Unless otherwise stated, all the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aspirated follicular fluids were collected into 50-mL Falcon tubes and allowed to settle for 30 min. The COC pellet was pipetted onto petri dishes containing 5 mL of Emcare Biofree Flushing Solution (ICPbio Reproduction, Spring Valley, Wisconsin, USA). COCs were washed twice in 3 mL Emcare solution and once in maturation medium before being matured for 24 h at 38.5 °C under 5% CO_2 in air with maximum humidity. Maturation medium (500 μL/well) was TCM-199 with glutamax-I (Gibco™; Invitrogen Corporation, Paisley, UK) supplemented with 0.25 mM Na pyruvate, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 ng/mL FSH (Puregon, Organon, Oss, Netherlands), 1 μg/mL β-estradiol (E-2257), and 10% heat-inactivated FBS (Gibco™, New Zealand).

2.1.3. In vitro fertilization

Following maturation, oocytes were washed twice in 3 mL Emcare solution and once with IVF-TALP [15] and transferred to fertilization wells. Frozen-thawed semen was washed twice with 4 mL sperm-TALP. Oocytes were fertilized for 20 h in IVF-TALP (500 μL/well) supplemented with 10 μg/mL of heparin and 2 mM of penicillinamine-hypotaurine-epinephrine at 38.5 °C under 5% CO_2 in air with maximum humidity, using 1×10^6 spermatozoa/mL as the final concentration.

2.1.4. In vitro culture

After fertilization, cumulus cells were removed from presumptive zygotes by vortexing for 90 s at medium speed. Presumptive zygotes were washed again twice in 3 mL Emcare solution and once in 3 mL of *in vitro* culture medium before being cultured in groups of 50 embryos in 50-μL droplets of G1/G2 media (Vitrolife, Göteborg, Sweden) supplemented with fatty acid-free BSA (4 mg/mL) and covered with Ovoil (Vitrolife, Göteborg, Sweden) at 38.5 °C under 5% O_2, 5% CO_2, and 90% N_2 with maximum humidity. The day of fertilization was Day 0. Cleavage rates were recorded 42 h post-fertilization. Blastocysts were evaluated and the integrity of the ZP was confirmed under a stereomicroscope. Blastocysts were collected on days 7 and 8 into Syngro® holding medium (Vetoquinol, Brisbane, Australia). The blastocyst percentage was calculated by summing the number of blastocysts collected on days 7 and 8 and calculating the percentage from the number of zygotes cultured in each experiment.

2.1.5. Handling of blastocysts

Blastocysts from a same culture well were divided into pools of 4–6 blastocysts. The pools were further divided into two handling lines. In the first line, the unwashed blastocyst pool in a sterile Eppendorf tube containing 100 μL Friis (F) broth [16] was placed in liquid nitrogen for 1 min to lyse the blastocysts. After lysis, 2.7 mL F broth was added to the tube and the contents were transferred to a tightly closed culture tube. The second treatment line was the IETS washes. A pool of four to six blastocysts was washed in five consecutive wells, each containing 500 μL of Syngro® holding medium, and then in two wells containing 0.25% trypsin in PBS, followed by five more washes in the holding medium. Washed embryos were further handled in the same way as unwashed embryos. During IVP, COCs, oocytes, and embryos were handled using sterile home-made micropipettes prepared from glass hematocrit capillaries. This led to the transfer of almost no liquids between wells, and thus the dilution effect was always more than the required minimum of 1:100.

2.2. Experimental design

2.2.1. Experiments

Maternal (experiments A1 and A2), paternal (experiments B1 and B2, semen naturally infected with *M. bovis*, and experiments C1 and C2, spiked semen used) and environmental infection (experiment D) routes were examined. An overview of the experiments is presented in Fig. 1.

2.2.2. M. bovis strains

Two wild-type *Mycoplasma bovis* strains were used for inoculation: strain 198, an isolate from bull semen obtained in 2015 [5], and strain 8970 (isolated from mastitis). Two concentrations, 10^3 CFU/mL and 10^6 CFU/mL, and both strains were used in the experiment involving cumulus–oocyte complexes (COCs).

2.2.3. Inoculation of COCs with M. bovis

In a preliminary test, COCs were exposed to either *M. bovis* strain 198 or 8970 at a concentration of either 10^3 CFU/mL or 10^6 CFU/mL in maturation medium for 24 h. After maturation, the COCs were washed three times with PBS–polyvinylpyrrolidone (PVP, 4 mg/mL), and cumulus cells (CCs) were removed using combined mechanical (vortexing) and trypsin (0.25%) treatment. After removal of CCs, the oocytes were washed three times with PBS–PVP and the ZP was removed. To do this, the oocytes were transferred to pre-incubated acidic Tyrode's solution (pH 2.1) at 37 °C for 60–90 s. Finally, the oocytes were washed three times with PBS–PVP. The maturation medium, CCs, and Tyrode's solution, including the dissolved ZPs and oocytes, were cultured.

2.2.4. Embryo production using COCs exposed to M. bovis (experiments A1 and A2)

Based on the culture results from the preliminary COC exposure testing, only the 10^6 CFU/mL *M. bovis* inoculation concentration and only strain 198 was used in all subsequent experiments. COCs were infected during the maturation step. Matured oocytes were fertilized with *M. bovis*-negative semen from an IVP-proven bull and cultured until the blastocyst stage. COCs not exposed to *M. bovis* were used as negative controls. Samples from follicular fluid, maturation medium, CCs, washed semen, fertilization medium, G1 and G2 culture media, cleaved zygotes, each of 12 wash media, and blastocysts (unwashed and IETS washes, including two trypsin washes) were collected for *M. bovis* culture.

2.2.5. Embryo production using semen naturally contaminated with M. bovis (experiments B1 and B2)

Matured oocytes were fertilized with naturally infected semen washed twice with sperm-TALP [5], lots 3 and 4 [5], and cultured until the blastocyst stage. Control oocytes were fertilized with semen from an IVP-proven bull. Samples for *M. bovis* culture were collected similarly to experiments A1 and A2.

2.2.6. ***Embryo production using spermatozoa inoculated with M. bovis (experiments C1 and C2)***

Washed semen from an IVP-proven bull was infected with *M. bovis* at 10^6 CFU/mL in sperm-TALP for 1 h before IVF. Matured oocytes were fertilized with the infected semen and cultured until the blastocyst stage. Control oocytes were fertilized with uninfected semen from the same bull. Samples for *M. bovis* culture were collected similarly to experiments A1 and A2.

2.2.7. Five-day-old embryos exposed to M. bovis

Embryos in G2 culture media were infected on day 5 with *M. bovis* by adding 2 μL of *M. bovis* in F broth under oil to give a final concentration of 10^6 CFU/mL, and the embryos were cultured until the blastocyst stage. G2 culture media and transferable embryos, as well wash media, were collected for *M. bovis* culture.

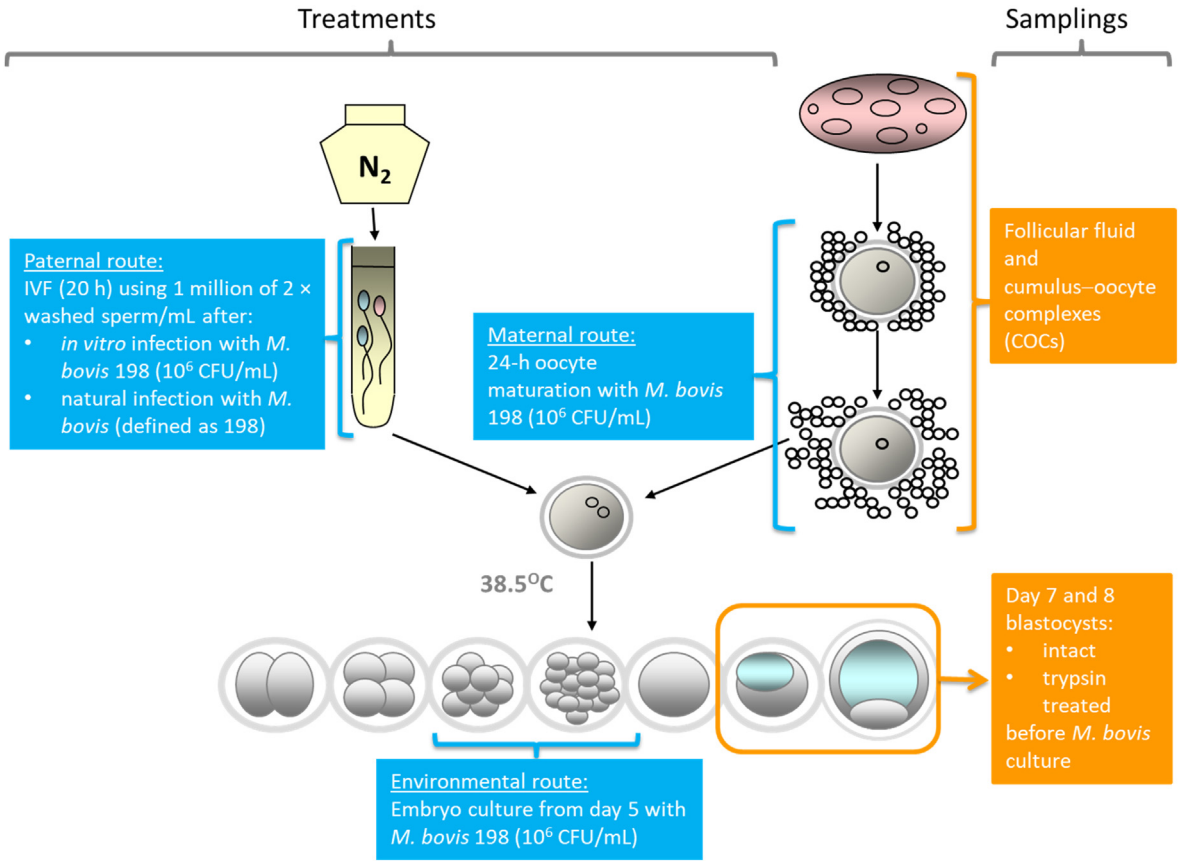

Fig. 1. Design of the experiments.

2.2.8. Detection of M. bovis

Samples of follicular fluid, maturation medium, CCs, fertilization medium, washed semen, culture media G1 and G2, all 12 wash media, and transferable embryos were collected for *M. bovis* culture. Embryos (4–6) were pooled into 100 μL of F broth [16] and lysed in liquid nitrogen for 1 min. Liquid samples were directly diluted 1:10 in F broth. Ten-fold dilutions up to 10^{-2} were prepared in F broth in tightly closed tubes and the tubes were incubated at 37 °C for 14 days. All broth cultures were examined for *M. bovis* using a real-time PCR assay targeting the 3′-terminal region of *opp*D gene (GenBank accession No. AF130119) of *M. bovis*, as described previously [17]. Briefly, primers PMB996-F (5′-TCAAGGAACCC-CACCAGAT) and PMB1066-R (5′-AGGCAA AGTCATTTCTAGGTGCAA) and the probe Mbovis1016 (FAM-TGGCAAACTTACCTATCG GTGACCCT-BHQ1) were used. The amplicon size is 71 bp. Plasmid pUC-18 was used as internal amplification control (IAC) according to Fricker et al. [18]. Primers pUC-IAC-F 5′-GCAGCCACTGGTAA-CAGGAT and pUC-IAC-R 5′-GCAGAGCGCAGATACCAAAT and pUC-IAC probe HEX-AGAGCGAGGTATGTAGGCGG-BHQ1 were used. F broth was prepared for real-time PCR as follows: 200 μL of medium was incubated for 15 min at 95 °C and centrifuged at 10 000×*g* for 5 min. The reaction volume was 22 μl and contained 11 μl iTaq™ Universal Probes Supermix (Bio-Rad), 2 μl template, 10 pmol each of the *M. bovis* and IAC primers, 4 pmol each probe and 1 μl pUC18. Amplifications were conducted using a Bio-Rad CFX96 cycler system and the following temperature–time profile: initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 30 s, and 60 °C for 60 s. Every 7th well was a non-template control and two different concentrations of *M. bovis* suspension were included in every run as positive controls. Sample was considered positive if Cq value was ≤37.

3. Results

3.1. Collected oocytes

All follicular fluids from slaughterhouse-collected ovaries used in these experiments were negative for *M. bovis*.

3.2. Inoculation of COCs with M. bovis

M. bovis was not detected in any of the samples when 10^3 CFU/mL exposure was used. At the high exposure level, *M. bovis* was detected in maturation medium and cumulus cells, whereas ZP and oocytes were negative (Table 1). Similar results were obtained for both strains studied.

3.3. Embryo production using COCs exposed to M. bovis (experiments A1 and A2)

M. bovis-contaminated maturation medium had no effect on embryo development (Table 2). *M. bovis* was recovered from maturation medium, CCs, and fertilization medium, whereas washed semen used for fertilization and all 12 wash media from each pool, as well as 25 pools of unwashed and 16 pools of IETS washed embryos, were negative. All 10 pools of control embryos (no exposure to *M. bovis* in maturation medium) were also negative (Table 3).

Table 1
Presence of *M. bovis* in cumulus-oocyte complexes after exposure to two *M. bovis* strains in maturation medium.

Sample	Strain and concentration			
	198		8970	
	10^6	10^3	10^6	10^3
Maturation medium	+	−	+	−
Cumulus cells	+	−	+	−
Zonae pellucidae	−	−	−	−
Oocytes	−	−	−	−

3.4. Embryo production using semen naturally contaminated with M. bovis (experiments B1 and B2) or semen inoculated with M. bovis (experiments C1 and C2)

Naturally contaminated semen had no detrimental effect on embryo development (Table 2). On the contrary, semen spiked with *M. bovis* for 1 h before insemination (experiment C1) showed lower cleavage and blastocyst rates compared to the same semen when uninfected ($p = 0.016$). In experiment C2, the spiked semen was accidently pipetted too forcefully during the wash step, which damaged the spermatozoa and led to decreased cleavage and a blastocyst rate of only 2% (Table 2).

When naturally infected washed semen was used for fertilization, *M. bovis* was recovered from washed semen in experiment B2 but not in experiment B1. Fertilization medium, all culture media, and 12 wash media from each pool as well as 21 pools of unwashed, 20 pools of IETS washed, and 11 pools of control embryos were all *M. bovis* negative (Table 3, experiments B1 and B2). When spiked semen was used, *M. bovis* was detected in samples from washed semen, CCs, and fertilization medium. G1 and G2 culture media, all 12 wash media, 9 pools of unwashed, 5 pools of IETS washed, and 19 control pools of embryos were all *M. bovis* negative (Table 3).

3.5. Five-day-old embryos exposed to M. bovis

No *M. bovis* could be detected in G2 culture media, 21 pools of unwashed and 11 pools of washed embryos, or any of the wash media (Table 3).

4. Discussion

In this *in vitro* bovine embryo production study, we infected both oocytes and spermatozoa with wild-type *M. bovis*, as well as using semen naturally containing *M. bovis* in fertilization. Contrary to earlier studies [6–8], we could not detect *M. bovis* in any of the embryo wash media or transferable embryos, despite an extensive number of samples being enriched in mycoplasma culture broth followed by real-time PCR detection.

The course of *M. bovis* infection in female genitalia is unknown. Hartmann et al. [19] and Hirth et al. [10] reported that in heifers, inoculation of *M. bovis* into the uterus or insemination with infected semen led to salpingitis, endometritis, and salpingoperitonitis with ovarian adhesions. Salary et al. [20] demonstrated that bovine follicular fluid is not always sterile and can occasionally contain bacteria. They did not analyze their samples for mycoplasma. There is some evidence indicating that bacteremia can occur in cattle during *M. bovis* infection [21]. This could lead to *M. bovis* contamination of ovaries collected in slaughterhouses. We attempted to mimic this situation by infecting oocytes in maturation medium. However, *M. bovis* appeared to only attach to cumulus cells, indicating that the potential occurrence of *M. bovis* in ovaries poses a low risk in *in vitro* embryo production.

Contrary to the low risk of slaughterhouse-collected oocytes being contaminated with *M. bovis*, infected semen might pose a real risk of *M. bovis* transmission in IVP. Several studies have demonstrated that mycoplasmas tightly adhere to the head and tail, and less tightly to the midpiece of spermatozoa [7,22], and are sometimes localized intracellularly [23,24]. Bielanski et al. [7] were able to establish that the swim-up procedure to separate spermatozoa

Table 2
Effect of *M. bovis* (*Mbo*) on embryonic development in experiments A1 to D.

Experiment	*Mbo* exposure CFU/ml	No. of zygotes cultured	Cleavage rate n (%)	Blastocysts n (%)	*p*
A1: Oocyte maturation with *Mbo*	10^6	302	249 (82.5)	99 (32.8)	0.794
	Control	72	59 (81.9)	22 (30.5)	
A2: Oocyte maturation with *Mbo*	10^6	281	229 (81.5)	103 (36.7)	0.495
	Control	79	61 (77.2)	34 (43.1)	
B1: Naturally infected semen	None	376	334 (88.8)	139 (37.0)	0.382
	Control	84	68 (81.0)	25 (30.9)	
B2: Naturally infected semen	None	238	188 (79.0)	85 (35.7)	0.713
	Control	74	64 (86.5)	29 (39.2)	
C1: Semen spiked with *Mbo*	10^6	396	242 (61.1)	66 (16.7)	0.016*
	Control	170	129 (75.9)	47 (27.6)	
C2: Semen spiked with *Mbo*	10^6	345	142 (41.2)	8 (2.3)	0.000**[b]
	Control	135	115 (85.2)	55 (40.7)	
D: 5-d embryos infected with *Mbo*	10^6	362[a]	Not calculated[a]	160 (44.2)	0.790
	Control	182[a]		84 (46.1)	

*$p \leq 0.05$, **$p \leq 0.000$.

[a] Cleavage rate not calculated in the experiment in which 5-d embryos were exposed to *M. bovis*.
[b] Forceful pipetting during washing damaged spermatozoa.

from seminal plasma and extender did not render spiked spermatozoa free of *M. bovis* or *M. bovigenitalium*. We washed all semen used in our IVP system twice before it was used in fertilization. *M. bovis* was detected in all washed semen lots, except in the first experiment (B1) with naturally contaminated semen. Thus, our results also support the finding of *M. bovis* adhering to spermatozoa. We previously demonstrated that the distribution of *M. bovis* in semen straws from a naturally infected bull was not equal: from the known positive lots, one out of five straws cultured and three out of four tested by direct PCR were negative [5]. Due to the limited number of straws available from the positive lots, we could not culture semen before washing, and cannot therefore conclude whether all semen straws used in the first experiment were actually negative, or more likely if the concentration of *M. bovis* was below the level of detection after washing. However, in the second experiment (B2), live *M. bovis* was detected in the semen after washing. Despite this, all transferable embryos were *M. bovis* negative, indicating that *M. bovis* is not transmitted in our IVP system, even if contaminated semen is used in fertilization.

Mammalian oocytes and embryos are protected until the hatching blastocyst stage by a porous acellular matrix, the ZP. Several studies have demonstrated that different mycoplasmas can adhere tightly to the pores in the outer surface of the ZP [6,7,22,25]. Bielanski et al. [6] exposed 7-day-old *in vivo*-produced bovine embryos to *M. bovis* for 24 or 48 h in Ham's F-10 culture medium without an oil cover. The ZP was mechanically removed after

Table 3
Isolation of *M. bovis* (*Mbo*) from samples taken from different stages during *in vitro* embryo production, as well as from unwashed and IETS-washed pools of IVP embryos. One pool contains 4–6 embryos.

Experiment	*Mbo* exposure CFU/ml	Embryo treatment	No. of tested pools	Total no. of embryos	Follicular fluid	Matura-tion medium	Cumulus cells	Washed semen	Fertiliza-tion medium	G1	G2	IETS wash media 1-12	Embryos
A1: Oocyte maturation with *Mbo*	10^6	Unwashed	12	58	–	+	+	–	+	–	–	nd	–
		IETS	8	41	–	+	+	–	+	–	–	–	–
	Control	Unwashed	4	22	–	–	–	–	–	–	–	nd	–
A2: Oocyte maturation with *Mbo*	10^6	Unwashed	13	53	–	+	+	–	+	–	–	nd	–
		IETS	8	40	–	+	+	–	+	–	–	–	–
	Control	Unwashed	6	34	–	–	–	–	–	–	–	nd	–
B1: Naturally infected semen	None	Unwashed	13	64	–	–	–	–	–	–	–	nd	–
		IETS	13	65	–	–	–	–	–	–	–	–	–
	Control	Unwashed	5	25	–	–	–	–	–	–	–	nd	–
B2: Naturally infected semen	None	Unwashed	8	40	–	–	–	+	–	–	–	nd	–
		IETS	7	35	–	–	–	+	–	–	–	–	–
	Control	Unwashed	6	29	–	–	–	–	–	–	–	nd	–
C1: Semen spiked with *Mbo*	10^6	Unwashed	6	30	–	–	+	+	+	–	–	nd	–
		IETS	5	25	–	–	+	+	+	–	–	–	–
	Control	Unwashed	9	47	–	–	–	–	–	–	–	nd	–
C2: Semen spiked with *Mbo*	10^6	Unwashed	3	13	–	–	+	+	+	–	–	nd	–
		IETS	0	0	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Control	Unwashed	10	55	–	–	–	–	–	–	–	nd	–
D: 5-d embryos infected with *Mbo*	10^6	Unwashed	21	105	–	–	–	–	–	–	–	nd	–
		IETS	11	55	–	–	–	–	–	–	–	–	–
	Control	Unwashed	17	84	–	–	–	–	–	–	–	nd	–

nd, not done.

exposure. Despite 10 washes, *M. bovis* was cultured from the ZP, indicating that the mycoplasmas were tightly attached to the surface of the ZP. Later, Bielanski et al. [7] produced *in vitro* embryos using *M. bovis*-spiked semen, similarly to our study. *M. bovis* was cultured from unwashed embryos and those that had been washed 10 times. This corroborated their previous finding that *M. bovis* is attached on the ZP and contrasted with our results. Due to the several experiments and high number of produced oocytes, mechanical removal of the ZP was not feasible in our study. In the COC infection experiment, we removed the ZP using Tyrode's solution and cultured this solution containing the dissolved ZP with negative results. However, it is highly likely that *M. bovis* attached to the ZP is destroyed in the highly acidic Tyrode's solution during the approximately 60–90 s it takes for the ZP to dissolve. Thus, we cannot directly confirm from our results the attachment of *M. bovis* to the ZP. However, we can conclude that we examined 55 pools containing altogether 258 transferable unwashed embryos with an intact ZP with negative results, demonstrating that at least in our IVP system, *M. bovis* did not survive until the 7–8-day blastocyst stage. The main difference between our IVP system and that of Bielanski et al. [7] is that they used a co-culture system in which a monolayer of cells is formed from cumulus cells in the maturation wells. We noticed in our COC infection studies that *M. bovis* appears to adhere to cumulus cells. The co-culture cell monolayer might have kept the *M. bovis* alive and even advanced the adherence of *M. bovis* to the ZP. In our study, we used sequential synthetic G1 and G2 culture media, as well as holding medium, for embryo washing, all of which contain hyaluronan. Among its many beneficial effects in *in vitro* embryo culture, hyaluronan can enhance the blastocyst production rate, as well as the post-transfer survival of embryos [26], but high-molecular-weight hyaluronan has also been shown to have an inhibitory effect on some microbes [27]. Further studies are needed to clarify the effect of hyaluronan on the survival of mycoplasmas in embryo production.

To our knowledge, natural exposure of embryos to *M. bovis* in the uterus of donor cows has never been shown, and neither are we aware of studies in which uterine collection medium has been tested for *M. bovis*. Bovine embryos arrive in the uterus approximately 4–5 days after fertilization and are collected for *in vivo* production two days later. To mimic this situation, we infected 5-day-old embryos with *M. bovis* pipetted into G2 media under oil and continued the culturing until day 7. *M. bovis* did not affect the blastocyst rate, and neither it was detected in G2 culture media, wash media, or in washed or unwashed embryos. However, our experimental design using embryo culture media covered with oil might not be fully equivalent to the conditions in the uterus.

ART allow the use of genetically elite animals for breeding and reduce the risk of disease transmission. However, we have previously established that AI with semen infected with *M. bovis* can pose a risk [5] and eliminating *M. bovis* from semen using antibiotics in extender is not a reliable method [28]. Contrary to this, the risk of *M. bovis* transmission in embryo transfer using *in vitro*-produced embryos appears to be very low.

5. Conclusions

We investigated the transmission of *M. bovis* in *in vitro* bovine embryo production. Altogether, 128 pools containing 624 ZP-intact transferable embryos were analyzed after oocytes, spermatozoa, or 5-day-old embryos were infected with wild-type *M. bovis* or naturally infected semen was used in fertilization. We could not demonstrate *M. bovis* in any of the embryo wash media or embryos (unwashed or washed) using sensitive enrichment culture followed by real-time PCR detection. We conclude that the risk of *M. bovis* transmission via *in vitro*-produced bovine embryos to recipient cows is low. Studies with specific *M. bovis* antibodies and confocal microscopy are needed to confirm the interaction of *M. bovis* with the bovine ZP. Moreover, further studies on the use of hyaluronic acid in controlling mycoplasmas in *in vitro* embryo production are warranted.

CRediT authorship contribution statement

Tarja Pohjanvirta: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Nella Vähänikkilä:** Conceptualization, Investigation, Data curation. **Mervi Mutikainen:** Conceptualization, Investigation. **Heli Lindeberg:** Conceptualization, Investigation, Writing – review & editing. **Sinikka Pelkonen:** Conceptualization, Funding acquisition, Writing – review & editing. **Jaana Peippo:** Conceptualization, Supervision, Writing – review & editing. **Tiina Autio:** Conceptualization, Supervision, Writing – review & editing.

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