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Accepted Manuscript

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PII: S0093-691X(17)30518-6

DOI: 10.1016/j.theriogenology.2017.10.033

Reference: THE 14316

To appear in: *Theriogenology*

Received Date: 19 April 2017

Revised Date: 23 October 2017

Accepted Date: 23 October 2017

Please cite this article as: Pellerin JL, Alsaleh A, Mermillod P, Souza-Fabjan JMG, Rodolakis A, Rousset E, Dubreil L, Bruyas JF, Roux C, Fieni F, Attachment of *Coxiella burnetii* to the *zona pellucida* of *in vitro* produced goat embryos, *Theriogenology* (2017), doi: 10.1016/j.theriogenology.2017.10.033.

ATTACHMENT OF *COXIELLA BURNETII* TO THE *ZONA PELLUCIDA* OF *IN VITRO* PRODUCED GOAT EMBRYOS

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Abstract

Previous work demonstrated that after infection of *in vivo* derived caprine embryos, *Coxiella burnetti* (*C. burnetii*) showed a strong tendency to adhere to the *zona pellicida* (ZP). To investigate the risk of *C. burnetii* transmission via embryo transfer of *in vitro*-produced goat embryos the aim of this study was, (i) to evaluate the ability of *C. burnetii* to adhere to the intact *zona pellicida* of *in vitro*-produced goat embryos and to determine by confocal microscopy the location of the bacteria, (ii) to test the efficacy of IETS recommended rules for the washing of bovine embryos to eliminate *C. burnetii*,

One hundred ZP-intact caprine embryos, produced *in vitro*, at the 8 to 16 cell stage, were randomly divided into 11 batches of eight to nine embryos. Nine batches were incubated for 18 hours with 10^9 *Coxiella*/ml of CbB1 strain (IASP, INRA Tours). The embryos then were recovered and washed in batches in 10 successive baths following the IETS guidelines. In parallel, two batches of embryos were subjected to similar procedures but without exposure to *C. burnetii*, to serve as the control group. One of the nine batches of infected embryos and one of the two non-infected control batches were separated to perform immunolabeling to locate the bacteria.

C. burnetii DNA was detected by C-PCR in all eight batches of infected embryos after 10 successive washings. However, bacterial DNA was not detected in the embryo control batch. The first five washing media of the infected group were consistently found to be positive and *Coxiella* DNA was detected in the wash bath up to the 10^{th} wash for two batches.

After immunolabeling, the observation of embryos under confocal microscopy allowed C. *burnetti* to be found on the external part of the *zona pellucida* without deep penetration.

This study clearly demonstrates that *C. burnetii*, after *in vitro* infection at 10^9 *Coxiella*/ml, stick strongly to the external part of the *zona pellucida* of *in vitro* produced caprine embryos without deap penetration and that the 10 washings protocol recommended by IETS to eliminate the pathogenic agents of bovine embryos is unable to eliminate these bacteria from *in vitro*-produced goat embryo .

***Keywords*:** *Coxiella burnetii*, goats, *in-vitro*-produced embryos, conventional PCR, real-time PCR, confocal microscopy.

1. Introduction

Coxiella burnetii (C. burnetii) is an obligate intracellular gram-negative bacterium from the *Coxiellaceae* family of the Gamma subdivision of *Proteobacteria* [1]. It is responsible for Q fever, a zoonosis with worldwide distribution that affects a wide range of domestic and wild mammals, birds, reptiles, fish and arthropods, as well as humans [2] [3]. Human infection is manifested by a febrile syndrome, pneumonia or hepatitis and is serious for pregnant women in whom it can cause miscarriage [3].

Goats, sheep and cattle are the most frequently infected species and represent the major sources of human infections [4] [5]. Infection in these species is usually subclinical. However, infection in sheep, goats and occasionally cattle can cause abortion in late gestation with stillbirths, premature deliveries and weak newborn animals [6, 7]. Following infection, animals shed *Coxiella burnetii* into the environment in large quantities through the products of parturition (placenta, lochies, fetal membranes and amniotic fluid) during normal delivery and abortions [8, 9], as well as through milk, urine, feces and semen [7] [10] [11]. The bacterium has the ability to resist difficult environmental conditions, probably due to the existence of small dense cell variants [3].

The transmission of infection between ruminants and to humans mainly occurs through the inhalation of contaminated aerosols [12], but may also occur via the digestive tract and tick vectors [13]. Sexual transmission has been demonstrated experimentally in mice [11] and found for a human couple, in whom

the bacterium was detected by PCR (Polymerase Chain Reaction) in the man's sperm[14].

Coxiella burnetii was isolated from semen of naturally infected bulls [15]. Sexual transmission is strongly suspected in domestic ruminants but remains unproven. The vertical transmission route has been demonstrated in ruminants; *C. burnetii* is often found in fetal organs following an abortion or premature delivery [16] [17].

Transmission by embryo transfer has been partially studied. In natural conditions, *C. burnetii* was identified with significant loads in the flushing media from the oviducts and the uterus of seropositive goats [18]. These results reveal the main source of *in utero* infection and indicate a risk factor for the transmission of *C. burnetii* during embryo transfer (ET). After experimental infection of *in vivo* derived goat embryos, the bacterium showed a strong tendency to adhere to the *zona pellicida* (ZP) and the washing procedure recommeanded by the International Embryo Transfer Society (IETS) failed to remove it [19]. It has been demonstrated that the ZP of *in vivo*-derived and *in vitro*-produced embryos are different, and the way embryos are produced modifies the interaction between ZP and various pathogens [20].

The distribution of the infection in small ruminants was well documented in 1982 in the south of France [21]. The last zoonotic episode in France was in the Drôme department in May 2014 with 46 confirmed human cases. The clinical signs in humans were pneumopathy. In livestock, some abortions occurred.

To investigate the risk of *C. burnetii* transmission via embryo transfer of *in vitro*-produced goat embryos, this study aims (i) to evaluate the ability of *C. burnetii* to adhere to the intact *zona pellicida* of *in vitro*-produced goat embryos and *to* determine by confocal microscopy the location of the bacteria, (ii) to test the efficacy of IETS-recommended rules for the washing of bovine embryos to eliminate *C. burnetii.*

2. Materials and methods

2.1 *Coxiella burnetii* strain

The *C. burnetii* strain CbC1phase I used in this study was originally isolated from the placenta of an aborted goat in a French herd (Allier, France). It was prepared and provided by IASP, INRA Tours, France. It had been isolated by intraperitoneal inoculation of three OF1 mice (8 weeks) with 0.2 ml of goat placenta homogenate. The mice were killed nine days post inoculation and their spleens were sampled and re-inoculated into specific pathogen-free embryonated hen eggs. After the 3rd passage in the chicken embryo, it was quantified, aliquoted and frozen at –80°C. This preparation contained 10^{11} bacteria/ml. To ensure purity, each aliquot used for exposures was diluted with 10 ml PBS then centrifuged twice for 15 min at 2,000 X g; the supernatant was recovered and centrifuged for 1 h at 13,000 X g. The pellet was diluted 1:100 in the exposure medium giving a final calculated concentration of 10^9 bacteria/ml.

130

131 **2.2 *In vitro* production of embryos**

132 Animal housing, care slaughtering as well as experiment and handling of
133 fabrics complied with the regulations in France in accordance with EU Directive
134 2010/63/EU and with good laboratory practices.

135

136 **2.2.1. Oocyte collection and maturation**

137 Ovaries were collected from adult goats at a local slaughterhouse and
138 transported within 2.5 h after collection to the laboratory at INRA Nouzilly in a
139 sterile saline solution maintained at a temperature of 30°C. Ovaries were washed
140 in warm saline (30°C) and oocytes were aspirated through a 18-1/2 gauge short
141 bevelled needle from all visible follicles between 2 and 5 mm in diameter into a
142 Falcon tube under gentle vacuum (30 mm Hg). The collection tube was filled in
143 advence with 5 ml of tissue culture medium (TCM-199) supplemented with 100
144 IU/ml heparin, 40 µg/ml gentamicin and 10 mM Hepes. Only oocytes surrounded
145 by multilayer unexpanded cumulus cells were used for *in vitro* maturation.

146 The cumulus oocyte complexes (COCs) were washed 12 times in TCM 199
147 supplemented with 40 µg/ml gentamicin, and then placed in 0.5 ml of maturation
148 medium (TCM 199 supplemented with 10 ng/ml epidermal growth factor (EGF)
149 and 100 µM cysteamine) in plastic 4-well Petri dishes (Nunc, Roskilde,
150 Denmark), each well containing 20–30 COCs. COCs were then incubated for 24 h
151 at 38.5°C in a humidified atmosphere of 5% CO_2 in the air. The cumulus
152 oophorus was completely removed by gentle pipetting and the oocytes were

153 washed 12 times in fertilization medium (synthetic oviduct fluid (SOF), without
154 BSA, but supplemented with 40 µg/ml gentamicin and 10% heat-inactivated
155 estrous sheep serum). Only oocytes with intact *zona pellucida* and without
156 cumulus cells were kept for *in vitro* fertilization (IVF).
157
158 **2.2.2. Semen collection and preparation**
159 Semen was collected from two bucks during the breeding season using an
160 artificial vagina and was pooled. Two straws of frozen semen were thawed for
161 each IVF trial.
162 Motile spermatozoa were separated by centrifugation (10 min, 900 g) on 2
163 ml of Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient
164 (45/90%). The supernatant was discarded, and the sperm pellet was re-suspended
165 in 2 ml of SOF without BSA but supplemented with 40 µg/ml gentamicin and 10
166 mM Hepes, and centrifuged (5 min, 900 g). The supernatant was discarded and
167 viable spermatozoa were diluted in the appropriate volume of fertilization medium
168 to achieve a final concentration of 1.10^7 spz/ml. Then the medium was incubated
169 for 30 min at 38.5°C in a humidified atmosphere of 5% CO_2 in the air to allow
170 capacitation.
171
172 **2.2.3. *In vitro* fertilization (IVF) - *in vitro* culture (IVC)**
173 Groups of 20 to 30 oocytes were transferred to 4-well Petri dishes
174 containing 450 µl of fertilization medium, with one oocyte-group for each
175 spermatozoa-group. Capacitated sperm (50 µl) were added to the fertilization

wells to give a final concentration of 1.10^6 spz/ml. Finally, spermatozoa and oocytes were co-incubated for 18 h at 38.5°C in a humidified atmosphere with 5% CO_2 in the air. The zygotes then were washed 12 times in the culture medium (SOF with 3 mg/ml BSA) to remove spermatozoa before being transferred to 4-well Petri dishes containing 25 µl of culture medium and covered with 700 µl of mineral oil. The zygotes were incubated for six days at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. After 48 h post-insemination, 10% (v/v) fetal calf serum (FCS) was added to the culture droplets. Four days after fertilization the embryos were transported to Oniris in tubes of 15 ml of culture medium at an ambient temperature. Only developed embryos, with 8 to 16-cells and an intact ZP, were selected using binocular microscope observation.

2.3 Experimental design

Four days after the IVF, 100 caprine embryos were randomly divided into 11 batches of eight to nine embryos.

Nine batches were placed in 1 ml of minimum essential medium (M2414, Sigma, France) supplemented with 10% FCS, 1% L-glutamine (2 mM final), 1% HEPES (0.01 M final), 2.5 µg/ml^{-1} Amphotericin B and 50 mg/ml Gentamycin and containing 10^9 *Coxiella*/ml of CbB1 strain (IASP, INRA Tours). After incubation for 18 h at 37°C in an atmosphere of 5% CO2, the embryos were recovered and washed in batches in 10 successive baths of a phosphate-buffered saline (PBS) and 5% FCS following the IETS guidelines.

After incubating for 18 h at 37°C, in an atmosphere of 5% CO2, the embryos were collected by batches and washed, through 10 successive washes in PBS, with 5% fetal calf serum, following the IETS guidelines [22]. A new sterile pipette was used for each successive wash; each wash corresponded to a dilution of 1:100 of the previous medium.

In parallel, two batches of embryos were subjected to similar procedures but without exposure to *C. burnetii* to serve as a control group.

One of the nine batches of infected embryos and one of the two non-infected control batches were separated for immunolabeling with the aim to locate the bacteria.

For the other nine batches of embryos (eight infected batches and one non-infected batch) the 10 wash baths were collected separately and centrifuged for 1 h at 13,000 x g. The washed embryos and the pellets of the 10 centrifuged wash baths were frozen at -20 °C prior to examination for evidence of *C. burnetii* using PCR.

2.4 Conventional PCR (C-PCR) procedure

DNA was extracted from the batches of embryos and the wash bath pellets using a "QIAamp Blood and Body Fluid Kit® Qiagen-France" in accordance with the manufacturer's instructions.

The detection of *Coxiella*-DNA was performed by amplifying a DNA fragment (337 pb) located in the transposon-like repetitive region (*IS1111*) gene, which is present in multiple copies in the *C. burnetii* genome, using two primers:

Trans B: 5'- CAAGAATGATCGTAACGATGCGC - 3' (349-371) bp, and Trans M: 5'- CTCGTAATCACCAATCGCTTCG - 3' (664-685 bp) (IASP, INRA, Tours, France). Three µl of extracted DNA were added to 22 µl of amplification solution. The latter contained 5 µl of ready-to-use solution containing all reagents required for PCR: HOT FIREPol® DNA polymerase, Proofreading enzyme, 5X Blend Master Mix Buffer, 7.5 mM $MgCl_2$, 2 mM dNTPs of each, BSA, Blue dye, Yellow dye, and a compound to increase sample density for direct loading (Solis BioDyne, Estonia), 0.75 µl of both Trans B and Trans M primer (20 µM Eurofins MWG Operon, Ebersberg, Germany), and 15.5 µl of distilled water DNase-RNase Free.

Amplification was performed in a thermal cycler (Mastercycler® Eppendorf) based on the following program: after initial denaturation at 94°C for 10 minutes, the samples were subjected to a series of 35 cycles of 30 second denaturation at 94°C, a 1 minute hybridization at 63°C, and a 3 minute elongation phase at 72°C. This was followed by a final elongation phase at 74°C for 10 minutes. Products were visualized by electrophoresis on 1.5% agarose gel. A positive control of *C. burnetii* (IASP, INRA, Tours, France) and a negative control (distilled water) were performed. Samples analyzed for *C. burnetii*-DNA using PCR were considered positive when a band of 337 bp, corresponding to the positive control, was visualized on agarose gel electrophoresis under UV light. The sensitivity of this PCR method has been proven in our laboratory (SSBR, Oniris, France); it detects 10 bacteria per ml of bacterial suspension (data not shown).

244

245

246

247 **2.5 Real-time PCR (RT-PCR) procedure**

248 Real-time PCR (RT-PCR) was used to amplify a DNA fragment of 76 bp
249 from the *icd* gene (isocitrate dehydrogenase), of which there is only one copy in
250 the *C. burnetii* genome. The following primers were used: forward, icd-439F:
251 CGT TAT TTT ACG GGT GTG CCA (439-459) and reverse, icd-514R: CAG
252 AAT TTT CGC GGA AAA TCA (494-514), with a TaqMan probe icd-464TM:
253 FAM-CAT ATT CAC CTT TTC AGG CGT TTT GAC CGT-TAMRA-T (464-
254 492).

255 DNase RNase-free water was used as a negative control. Standard series
256 containing: 2.10^3, 2.10^4, 2.10^5, 2.10^6, 2.10^7 *C. burnetii*/ ml (IASP, INRA, Tours,
257 France) were extracted using the QIAamp DNA mini kit ® (Qiagen-France) and
258 used as a positive control. Five microliters of extracted DNA were added to 20 µl
259 of RT-PCR reaction mix. The latter was composed of 12.5 µl TaqMan® Universal
260 Master Mix II (Applied Biosystems, USA), 2.5 µl of a mixture of forward and
261 reverse primers (0.3 µM Eurofins MWG Operon, Ebersberg, Germany), 0.25 µl
262 TaqMan probe (50 nM Eurofins MWG Operon, Ebersberg, Germany). Water was
263 added to make a final volume of 20 µl. All RT-PCR reactions were performed in
264 duplicate in an ABIPRISM® Sequence Detection System 7300 (Applied
265 Biosystems) as follows: after 2 minutes at 50°C and 10 minutes at 95°C, the
266 samples were subjected to a series of 40 cycles comprising 15 seconds at 95°C

and 30 seconds at 60°C. Data were analyzed with the corresponding software. The *C. burnetii* titers in the samples were calculated in comparison with a standard curve obtained from a standard serial dilution of the bacteria.

2.6 Immunolabeling and confocal microscopy

The detection of *C. burnetii* was made using immunofluorescence labeling and analysed by confocal microscopy (microscope C1, Nikon, Champigny, France).

2.6.1 Immunolabeling

Initially the infected and non-infected embryos were fixed in 4% paraformaldehyde for 1 h at room temperature. They then were washed three times in phosphate buffered-saline (PBS) containing 0.2% PVP (polyvinylpyrrolidone) and transferred in a solution of PBS supplemented with 10% FCS (Fetal Calf Serum) and 0.2% Triton X-100 (Solution 1) for 1 h at room temperature.

After a first blocking step consistint of an incubation of 30 mn with a blocking buffer at room temperature (5% of normal donkey serum diluted in phosphate-buffered saline), the embryos were incubated 1 hour at 37°C with the primary antibody, a rat polyclonat anti CbC1 prepared in our laboratory.

After three careful washings in PBS buffer, the embryos were incubated for 1 h at 37°C with the secondary antibody, Alexa fluor 488, donkey anti-rat serum (A 21208- Invitrogen), diluted at 1/400.

After washing twice in PBS-PVP, labeled embryos were fixed with PFA 4% for 15 minutes.

Infected and non-infected embryos were labeled by a CbC1 non-immune serum, to control the specificity of the immunolabeling. Immunolabeled samples were mounted in Lab-Tek chambered coverglasses in PBS-PVP to preserve their structure.

2.6.2 Confocal microscopy

Confocal imaging was performed on an inverted Nikon TE-2000 laser scanning confocal microscope C1 equipped with a 488 nm argon laser and a 633 nm HeNE laser (Nikon, Champigny France). Z stacks were performed throughout the thickness of the embryos. 3D reconstructions were made from stacks using Fiji software.

3. Results

C. burnetii DNA was detected by C-PCR in all eight batches of infected ZP-intact embryos after 10 successive washings, following the IETS protoco1 (Table 1). However, bacterial DNA was not detected in the embryo control batches. The first five washing media of the infected group were consistently

313 found to be positive and Coxiella DNA was detected in the wash bath up to the
314 10^{th} wash for two batches.

315 All of the exposure baths and, after the 10 wash cycles, the batches of
316 embryos were tested using RT-PCR to quantify the bacterial load. The bacterial
317 load in the exposure baths ranged from 3.5 to 3.6 x 10^8 bacteria/ml with an
318 average of 3.55±0.05 x 10^8 bacteria/ml. The bacterial load for embryos after the
319 10 wash baths was less than 10^4 bacteria/ml (Table 1).

320 After immunolabeling, the observation of embryos under confocal
321 microscopy allowed C. *burnetti* to be found against the external part of the *zona*
322 *pellucida* without deep penetration (Figure 1). The presence of *C. burnetii* was
323 seen on the surface of the *zona pellucida,* with bacterial loads differing from one
324 embryo to another in the same batch.

325

326 **4. Discussion**

327

328 This study clearly demonstrates that *C. burnetii* stick strongly to the *zona*
329 *pellucida* of *in vitro*-produced caprine embryos after *in vitro* contamination.
330 Furthermore, the routine procedures proposed by IETS are not effective for
331 removing the bacteria from ZP-intact caprine embryos derived *in vivo* and
332 infected *in vitro*. For two batches of embryos, all 10 wash baths were positive for
333 *C. burnetii*, suggesting that a huge quantity of bacteria were attached to the *zona*
334 *pellucida* and were progressivelly released.

Recently, we demonstrated that *C. burnetii* binds strongly to the ZP of *in vivo*-derived goat embryos after *in vitro* infection [19] and to ZP of *in vitro*-produced bovine embryos after *in vitro* infection [23]. The ZP of intra-follicular oocytes appears to differ from that of ovulated ova; as a consequence ZP from *in vitro*-produced embryos cannot interact with infectious agents in the same way as those of embryos that are produced *in vivo* [24, 25]. It is therefore inadvisable to apply the same sanitary guidelines for *in vitro*-produced embryos as those recommended for *in vivo*-produced embryos [26]. It was shown that the use of *in vitro*-produced embryos increases the probability of adhesion of a number of pathogenic agents to the ZP of these embryos [20, 27].

In order to see and locate the bacteria on the contaminated caprine embryos, we used immunofluorescence techniques. The observation, by confocal microscopy, of the contaminated caprine embryos demonstrated that *C. burnetii* were present, with different bacterial loads, in the external part of the ZP without deap penetration.

This fixation may be due to interaction between lipopolysaccharide and membrane proteins of *C. burnetii* and proteins on the surface of the ZP [3]. The difference of bacterial load may be due to differences in the ultrastructure of the ZP that should be studied further by electron microscopy [27]. At the embryo cell level, this experiment did not directly examine the potential existence of receptors but suggests an unknown strong adherence. Purification of the inoculum by dilution and differential centrifugation allowed us to eliminate the role of egg protein [28]; the inoculum used in this study was ovoculture-*Coxiella. C. burnetii*

is pleomorphic with approximate dimensions of 0.3 by 1 µm and an envelope similar to that of gram-negative bacteria. It has two phases: I and II; this phase variation is due to differences in surface antigens [28]. Phase I *Coxiella* have LPS that completely hide the surface proteins of the external membrane. The role of this LPS in the attachment of ZP is highly plausible [29].

The *zona pellucida* of mammalian embryos is made of three glycoproteins (ZP1, ZP2 and ZP3), building a loose network and presenting on the surface a large number of canalicular pores [30]. The dimension of these pores, for example 182 nm for bovine embryos, influences the fixation and penetration of small size pathogenic agents [31]. Despite the presence of these pores, we demonstrate here that *C. burnetii* is present in the external part of the ZP without deap penetration. This property should permit the use of an enzymatic treatment to eliminate these bacteria on the surface of the embryo.

The efficacy of the ZP as a barrier to different pathogenic agents has been demonstrated for a number of agents. However, some of these pathogenic agents are able to penetrate the ZP or stick firmly to the surface and then resist washings [20].

In previous studies of bovine embryos exposed *in vitro* to *Mycoplasma bovis, Mycoplasma bovigenitalium, Mycobacterium avium,* and after carrying out the washing procedures, the bacteria were isolated from all of the ZP-intact embryo batches [32, 33, 34]. In other studies, the interaction between *Leptospira hardjobovis* and *in vitro*-produced bovine embryos after an *in vitro* infection, and after the IETS washing procedures, was examined with electronic microscopy

transmission. The presence of the bacteria was observed on the surface, in the pores of the ZP, in the intercellular spaces, on the vitellin and in the embryonic cells [27]. Other studies of mice infected *in vitro* with *C. burnetii* proposed the existence of specific receptors on the head of spermatozoids that fixed the bacteria [11, 15]. These studies showed clearly that adherence to the ZP depends on the structure of the ZP and on the outer membrane of the bacteria, and that transmission by embryo transfer is possible.

In conclusion, this study demonstrates that *C. burnetii* stick strongly to the external part of the ZP of *in vitro* produced caprine embryos without deap penetration. The ten washings protocol recommended by IETS [26] to eliminate the pathogenic agents of bovine embryos is unable to eliminate these bacteria.The presence of *C. burnetii* was observed on the surface of the ZP, with the bacterial load differing from one embryo to another in the same batch. This difference of load may be due to the ZP ultrastructure which would be interesting to analyse in the future by electron microscopy. Nevertheless, the finding of *C. burnetii* DNA by C-PCR does not imply that the bacteria found are still infective.

Acknowledgements

The authors would like to thank Véronique Blouin, Sylvie Saleun and the entire technical service from UMR649 Institut de Recherche Thérapeutique - IRT1-INSERM, 8 Quai Moncousu BP 70721-44007 Nantes cedex 01, France. The

confocal microscopy analysis was performed from the fluorescence bio-imaging expertise of the APEX platform UMR703 INRA Oniris, Nantes.

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532 TABLE AND FIGURE

533

534 **Table 1:** Detection of *Coxiella burnetii* in successive embryo wash baths and
535 batches of infected *zona pellucida*-intact 8 to 16 cell embryos after 10 wash
536 cycles, using C-PCR and quantification of *Coxiella burnetii* in embryo exposure
537 baths and in batches of infected *zona pellucida*-intact 8 to 16 cell embryos by RT-
538 PCR.

539

540

541 **Figure 1:** Immunofluorescent detection of *Coxiella burnetii* in *in vitro* produced
542 goat embryos after *in vitro* infection with 109 *C. burnetii* /ml for 18 hours.
543 *Coxiella burnetii* was localised at the surface of the embryo (see arrow), in the
544 external part of the *zona pellucida* of the contaminated caprine embryos without
545 deep penetration.
546 A - Negative control: A1 observation with brighfield micoscope; A2: observation
547 with confocal microscope without primary antibody. B - Coxiella detection: B1
548 observation with brightfield microscopem. B2: observation confocal microscope
549 after immunolabelling. Observation at objectif 60x2. Scale bar, 10 µm.

550

551

552 TABLE

553

554 **Table 1:** Detection of *Coxiella burnetii (C. burnetii)* in successive embryo wash
555 baths and batches of infected *zona pellucida*-intact 8 to 16 cell embryos after 10
556 wash cycles, using C-PCR and quantification of *Coxiella burnetii* in embryo
557 exposure baths and in batches of infected *zona pellucida*-intact 8 to 16 cell
558 embryos by RT-PCR.

559

Batches of embryos	Exposure bath (*Coxiella*/ml) (RT-PCR)	Last positive wash for *C. burnetii* (C-PCR)	Batch of embryos After 10 wash baths	
			Detection of *C. burnetii* (C-PCR)	Quantification of *C. burnetii* (RT-PCR)
1	$3.5x10^8$	6	Positive	$2.3x10^3$
2	$3.5x10^8$	8	Positive	$3.4x10^3$
3	$3.5x10^8$	6	Positive	$1.2x10^3$
4	$3.6x10^8$	5	Positive	$4.5x10^3$
5	$3.6x10^8$	7	Positive	$2.6x10^3$
6	$3.6x10^8$	10	Positive	$1.8x10^3$
7	$3.6x10^8$	5	Positive	$5.6x10^3$
8	$3.6x10^8$	10	Positive	$3.2x10^3$

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563

564 **Figure 1 :** Immunofluorescent detection of *Coxiella burnetii* in *in vitro* produced
565 goat embryos after *in vitro* infection with 10^9 *Coxiella/*ml for 18 hours.
566 *Coxiella burnetii* was localised at the surface of embryo (see arrow), in the
567 external part of the *zona pellucida* of the contaminated caprine embryos without
568 deep penetration.
569 A - Negative control: A1 observation with brighfield micoscope; A2: observation
570 with confocal microscope without primary antibody. B - Coxiella detection: B1
571 observation with brightfield microscopem. B2: observation confocal microscope
572 after immunolabelling. Observation at objectif 60x2. Scale bar, 10 µm.

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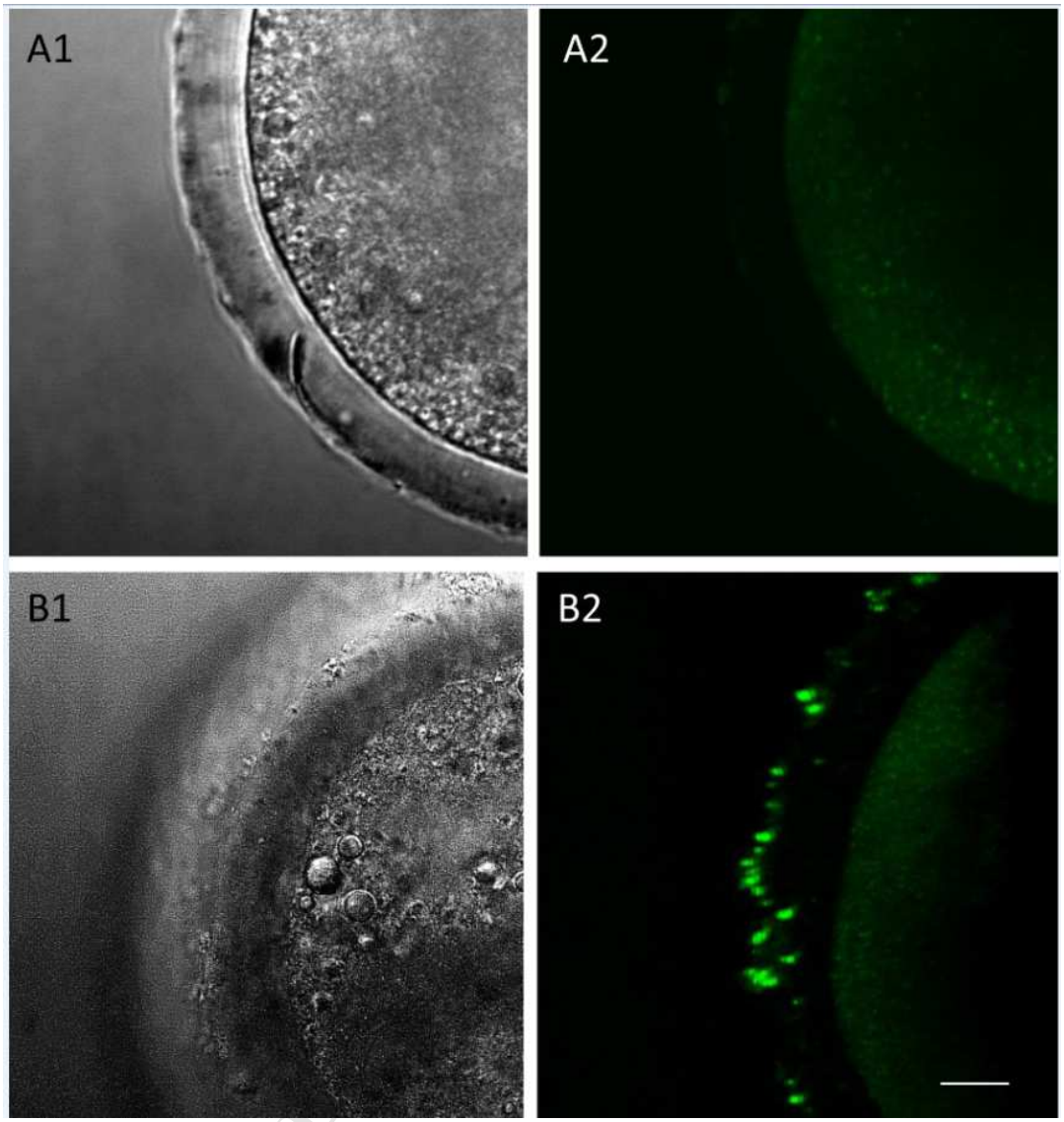

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