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1 **ATTACHMENT OF *COXIELLA BURNETII* TO THE ZONA** 2 ***PELLUCIDA* OF *IN VITRO* PRODUCED GOAT EMBRYOS**

3
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21

22

23 Abstract

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

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

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

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

49 This study clearly demonstrates that *C. burnetii*, after *in vitro* infection at
50 10^9 *Coxiella*/ml, stick strongly to the external part of the *zona pellucida* of *in vitro*
51 produced caprine embryos without deep penetration and that the 10 washings
52 protocol recommended by IETS to eliminate the pathogenic agents of bovine
53 embryos is unable to eliminate these bacteria from *in vitro*-produced goat embryo
54 .

55

56

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

59

60

61 1. Introduction

62

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

70 Goats, sheep and cattle are the most frequently infected species and
 71 represent the major sources of human infections [4] [5]. Infection in these species
 72 is usually subclinical. However, infection in sheep, goats and occasionally cattle
 73 can cause abortion in late gestation with stillbirths, premature deliveries and weak
 74 newborn animals [6, 7]. Following infection, animals shed *Coxiella burnetii* into
 75 the environment in large quantities through the products of parturition (placenta,
 76 lochies, fetal membranes and amniotic fluid) during normal delivery and abortions
 77 [8, 9], as well as through milk, urine, feces and semen [7] [10] [11]. The
 78 bacterium has the ability to resist difficult environmental conditions, probably due
 79 to the existence of small dense cell variants [3].

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

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

86 *Coxiella burnetii* was isolated from semen of naturally infected bulls [15].
87 Sexual transmission is strongly suspected in domestic ruminants but remains
88 unproven. The vertical transmission route has been demonstrated in ruminants; *C.*
89 *burnetii* is often found in fetal organs following an abortion or premature delivery
90 [16] [17].

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

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

106

107

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

114

115 2. Materials and methods

116

117 2.1 *Coxiella burnetii* strain

118 The *C. burnetii* strain CbC1phase I used in this study was originally
 119 isolated from the placenta of an aborted goat in a French herd (Allier, France). It
 120 was prepared and provided by IASP, INRA Tours, France. It had been isolated by
 121 intraperitoneal inoculation of three OF1 mice (8 weeks) with 0.2 ml of goat
 122 placenta homogenate. The mice were killed nine days post inoculation and their
 123 spleens were sampled and re-inoculated into specific pathogen-free embryonated
 124 hen eggs. After the 3rd passage in the chicken embryo, it was quantified, aliquoted
 125 and frozen at -80°C. This preparation contained 10¹¹ bacteria/ml. To ensure
 126 purity, each aliquot used for exposures was diluted with 10 ml PBS then
 127 centrifuged twice for 15 min at 2,000 X g; the supernatant was recovered and
 128 centrifuged for 1 h at 13,000 X g. The pellet was diluted 1:100 in the exposure
 129 medium giving a final calculated concentration of 10⁹ 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 advance 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% CO₂ 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% CO₂ 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

176 wells to give a final concentration of 1.10^6 spz/ml. Finally, spermatozoa and
 177 oocytes were co-incubated for 18 h at 38.5°C in a humidified atmosphere with 5%
 178 CO₂ in the air. The zygotes then were washed 12 times in the culture medium
 179 (SOF with 3 mg/ml BSA) to remove spermatozoa before being transferred to 4-
 180 well Petri dishes containing 25 µl of culture medium and covered with 700 µl of
 181 mineral oil. The zygotes were incubated for six days at 38.5°C in a humidified
 182 atmosphere of 5% O₂, 5% CO₂, and 90% N₂. After 48 h post-insemination, 10%
 183 (v/v) fetal calf serum (FCS) was added to the culture droplets. Four days after
 184 fertilization the embryos were transported to Oniris in tubes of 15 ml of culture
 185 medium at an ambient temperature. Only developed embryos, with 8 to 16-cells
 186 and an intact ZP, were selected using binocular microscope observation.

187

188 **2.3 Experimental design**

189 Four days after the IVF, 100 caprine embryos were randomly divided into
 190 11 batches of eight to nine embryos.

191 Nine batches were placed in 1 ml of minimum essential medium (M2414,
 192 Sigma, France) supplemented with 10% FCS, 1% L-glutamine (2 mM final), 1%
 193 HEPES (0.01 M final), 2.5 µg/ml⁻¹ Amphotericin B and 50 mg/ml Gentamycin
 194 and containing 10⁹ *Coxiella*/ml of CbB1 strain (IASP, INRA Tours). After
 195 incubation for 18 h at 37°C in an atmosphere of 5% CO₂, the embryos were
 196 recovered and washed in batches in 10 successive baths of a phosphate-buffered
 197 saline (PBS) and 5% FCS following the IETS guidelines.

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

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

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

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

213

214 **2.4 Conventional PCR (C-PCR) procedure**

215 DNA was extracted from the batches of embryos and the wash bath pellets
 216 using a “QIAamp Blood and Body Fluid Kit[®] Qiagen-France” in accordance with
 217 the manufacturer’s instructions.

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

221 Trans B: 5'- CAAGAATGATCGTAACGATGCGC - 3' (349-371) bp, and Trans
 222 M: 5'- CTCGTAATCACCAATCGCTTCG - 3' (664-685 bp) (IASP, INRA,
 223 Tours, France). Three µl of extracted DNA were added to 22 µl of amplification
 224 solution. The latter contained 5 µl of ready-to-use solution containing all reagents
 225 required for PCR: HOT FIREPol[®] DNA polymerase, Proofreading enzyme, 5X
 226 Blend Master Mix Buffer, 7.5 mM MgCl₂, 2 mM dNTPs of each, BSA, Blue dye,
 227 Yellow dye, and a compound to increase sample density for direct loading (Solis
 228 BioDyne, Estonia), 0.75 µl of both Trans B and Trans M primer (20 µM Eurofins
 229 MWG Operon, Ebersberg, Germany), and 15.5 µl of distilled water DNase-RNase
 230 Free.

231 Amplification was performed in a thermal cycler (Mastercycler[®]
 232 Eppendorf) based on the following program: after initial denaturation at 94°C for
 233 10 minutes, the samples were subjected to a series of 35 cycles of 30 second
 234 denaturation at 94°C, a 1 minute hybridization at 63°C, and a 3 minute elongation
 235 phase at 72°C. This was followed by a final elongation phase at 74°C for 10
 236 minutes. Products were visualized by electrophoresis on 1.5% agarose gel. A
 237 positive control of *C. burnetii* (IASP, INRA, Tours, France) and a negative
 238 control (distilled water) were performed. Samples analyzed for *C. burnetii*-DNA
 239 using PCR were considered positive when a band of 337 bp, corresponding to the
 240 positive control, was visualized on agarose gel electrophoresis under UV light.
 241 The sensitivity of this PCR method has been proven in our laboratory (SSBR,
 242 Oniris, France); it detects 10 bacteria per ml of bacterial suspension (data not
 243 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.

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

293 After washing twice in PBS-PVP, labeled embryos were fixed with PFA
 294 4% for 15 minutes.

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

299

300 **2.6.2 Confocal microscopy**

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

306

307 **3. Results**

308

309 *C. burnetii* DNA was detected by C-PCR in all eight batches of infected
 310 ZP-intact embryos after 10 successive washings, following the IETS protocol
 311 (Table 1). However, bacterial DNA was not detected in the embryo control
 312 batches. 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 10th wash for two batches.

All of the exposure baths and, after the 10 wash cycles, the batches of embryos were tested using RT-PCR to quantify the bacterial load. The bacterial load in the exposure baths ranged from 3.5 to 3.6 x 10⁸ bacteria/ml with an average of 3.55±0.05 x 10⁸ bacteria/ml. The bacterial load for embryos after the 10 wash baths was less than 10⁴ bacteria/ml (Table 1).

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

4. Discussion

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

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

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

350 This fixation may be due to interaction between lipopolysaccharide and
 351 membrane proteins of *C. burnetii* and proteins on the surface of the ZP [3]. The
 352 difference of bacterial load may be due to differences in the ultrastructure of the
 353 ZP that should be studied further by electron microscopy [27]. At the embryo cell
 354 level, this experiment did not directly examine the potential existence of receptors
 355 but suggests an unknown strong adherence. Purification of the inoculum by
 356 dilution and differential centrifugation allowed us to eliminate the role of egg
 357 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 deep 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 spermatozooids 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 deep 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.

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TABLE AND FIGURE

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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.

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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 brightfield microscope; A2: observation
 547 with confocal microscope without primary antibody. B - *Coxiella* detection: B1
 548 observation with brightfield microscope. B2: observation confocal microscope
 549 after immunolabelling. Observation at objectif 60x2. Scale bar, 10 μ m.

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552 TABLE

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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.

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

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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 brightfield microscope; A2: observation
 570 with confocal microscope without primary antibody. B - *Coxiella* detection: B1
 571 observation with brightfield microscope. B2: observation confocal microscope
 572 after immunolabelling. Observation at objective 60x2. Scale bar, 10 μ m.

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