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Detection and quantification of *Cryptosporidium* oocysts in environmental surfaces of an Equine Perinatology Unit

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Detection and quantification of *Cryptosporidium* oocysts in environmental surfaces of an Equine Perinatology Unit

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17 **Abstract**

18 The presence of *Cryptosporidium* in institutions such as veterinary teaching hospitals, where students
19 and staff are in frequent contact with animals, could represent a serious public health risk. In this
20 study the detection and quantification of the *Cryptosporidium* oocysts present on the environmental
21 surfaces of an Equine Perinatology Unit (EPU) were investigated. During 3 foaling seasons 175
22 samples obtained by swabbing an area of the floor and walls of boxes and utility rooms of EPU with
23 sterile gauze, in 3 different moments. Samples were collected at the end of foaling season (July), after
24 washing procedures (September) and after washing and disinfecting procedures, at the beginning of
25 a new foaling season (December). All the samples were subjected to nested-PCR, followed by
26 genotyping and sub-typing methods and to qPCR, allowing the oocyst quantification.
27 *Cryptosporidium* spp. was detected in 14 samples, of which 11 were from walls and three were from
28 floors. The highest number of oocysts was found in a sample collected from the floor of one utility
29 room used for setting up therapies and treatments. In most cases, oocyst numbers, estimated by qPCR,
30 were reduced or eliminated after washing and disinfecting procedures. The genotyping and sub-
31 typing methods allowed identification of 2 subtypes of *C. parvum* (IIaA15G2R1 and IIdA23G1) and
32 1 of *Cryptosporidium* horse genotype (VIaA15G4) that were described in foals hospitalized at the
33 EPU in the same years. The results of the present study show that qPCR can be used to evaluate
34 *Cryptosporidium* contamination of environmental surfaces of a veterinary teaching hospital and the
35 efficacy of the disinfection procedures.

36

37 Key words: *Cryptosporidium*, nested-PCR, qPCR, environmental surfaces, equine.

38

39 **1. Introduction**

40 *Cryptosporidium* species are common food and water-borne protozoa affecting a wide variety of
41 domestic and wild animals as well as humans. In horses, cryptosporidiosis was first described in
42 immunodeficient Arabian foals by Snyder et al. (1978) and subsequently reported in
43 immunocompetent horses worldwide (Santin et al., 2013). The latter study showed that foals were
44 more susceptible to infection by *Cryptosporidium* spp. than older animals. Fully infectious,
45 environmentally resistant oocysts are excreted in the host's faeces. *Cryptosporidium* oocysts have
46 shown considerable resistance against the effects of most commercial disinfectants and those with
47 greater efficacy needed prolonged exposure times, which is not always obtainable in the field.
48 Commercial disinfectant containing hydrogen peroxide, chlorine dioxide and ammonia appear the
49 most effective (Fayer et al., 2008).

50 The ability of *Cryptosporidium* to break through water treatment barriers and to cause large-scale
51 outbreaks has had huge impact on the water industry and its regulation, so it has been used as a
52 reference pathogen for the faecal-orally transmitted protozoan in the design and implementation of
53 the WHO Guidelines for drinking Water Quality (Chalmers and Katzer, 2013).

54 While many studies focused on detection of *Cryptosporidium* spp. in water have been performed
55 (Castro-Hermida et al., 2009; Smith and Nichols, 2010; Feng et al., 2011; Khaldi et al., 2011; Nolan
56 et al., 2013), only two surveys were published on the detection of *Cryptosporidium* spp. on
57 environmental surfaces, in particular in a newborn calves herd in California (USA) (Atwill et al.,
58 1998) and in a swimming pool in Sicily (Italy) (Maida et al., 2008).

59 Quantitative real-time PCR (qPCR) has proven to be a user-friendly and fast approach to detect and
60 enumerate microorganisms in various environmental samples (Haugland et al., 2005). Many studies
61 proposed *Cryptosporidium*-specific qPCR as a technique for detecting *Cryptosporidium* oocysts in
62 water samples (Fontaine and Guillot, 2002; Masago et al., 2006; Staggs et al., 2013) and recently in
63 soil and vegetables (Hong et al., 2014). In addition, qPCR has the advantage that the level of oocyst
64 contamination can be easily quantified. Environmental contamination with *Cryptosporidium* is of

65 particular concern in institutions such as veterinary teaching hospital, where students, staff and
66 animals are in close contact to each other. This represents a risk for the possible transmission to both
67 healthy animals and humans, as already described in other studies (Anderson et al., 1982; Pohiola et
68 al., 1986; Levine et al 1988; Reif et al., 1989; Konkle et al., 1997; Preiser et al., 2003; Gait et al.,
69 2008; Kinross 2015).

70 The purpose of this study was to detect and quantify the oocysts on the environmental surfaces of the
71 Equine Perinatology Unit “Stefano Belluzzi” (EPU), of the Department of Veterinary Medical
72 Sciences, *Alma Mater Studiorum* -University of Bologna using nested-PCR and qPCR. In this
73 research two molecular tools were compared and the risk associated with environmental
74 contamination and the efficacy of cleaning and disinfection procedures were evaluated, in the
75 building (EPU) where, in the same period, Galuppi et al. (2015) reported a prevalence of 37.8% for
76 *Cryptosporidium* in faecal specimens collected from foals.

77

78 **2. Material and Methods**

79 **2.1 Location**

80 EPU houses 13 boxes, 5 of which are dedicated to neonatal intensive therapy. In addition, two
81 paddocks and two utility rooms, one used for setting up therapies and treatments (A) and one for drug
82 and medical supplies storage (B), complete the unit (Fig. 1). The floor of the unit is made of concrete.
83 The walls of all the 13 boxes are made of plaster, 11 boxes (number: 3, 4, 5, 6, 7, 8, 9,10, 11, 12,13)
84 present a wooden wall and 5 boxes (number: 1, 2, 3, 5,7) presented a rubber mat leaning on a wall.
85 At the EPU, during each foaling season (from January to July) pregnant mares and foals affected by
86 one or more diseases were hospitalized for variable lengths of time and, at the discharge, routine
87 disinfection procedures are performed as described by Galuppi et al. (2015). Briefly, feces are
88 removed and straw replaced every day from the boxes; at animal discharge, the straw is completely
89 removed and watering devices emptied; the organic debris is removed from walls and floors using a
90 pressure washer and, once dried, the boxes are coated with 2% Steramine G solution left for 48 H;

91 finally Virkon-S 1% solution is sprayed and left for at least 1 h. Moreover, at the end of the foaling
92 season, two more steps of disinfection practices are carried out. In September, the walls and floors of
93 the boxes are cleaned with a pressure washer (about 100 °C and 100 bar); in December, after a new
94 cleaning with a pressure washer, the dried boxes are wet with Steramine G[®] solution (10% quaternary
95 ammonio compound and 2.5% nonionic surfactant) left for 48 hours; finally 1% Virkon-S[®] solution
96 (21.4% potassium peroxymonosulfate and 1.50 % sodium chloride) was sprayed and left for at least
97 one hour.

98

99 **2.2 Sampling**

100 From September 2011 to December 2013 (including 3 foaling seasons), samples were collected by
101 rubbing a sterile gauze over an area of approximately 60×60 cm of the floor and wall of boxes and
102 on the floor of utility rooms. Each year, sampling was performed as follow: in July (except 2011), at
103 the end of the foaling season; in September, after washing procedure; in December, after washing
104 and disinfecting procedure, before the beginning of the new foaling season.

105 One hundred seventy-five samples were collected: 70 from boxes floor, 10 from utility rooms floor
106 and 95 from boxes wall (54 samples from plastered walls, 22 from walls covered with rubber mats
107 and 19 from wooden walls).

108 **2.3 Molecular analysis**

109 Each gauze was placed into a 50 ml tube with 9 ml of PBS and left overnight. The gauze was hung
110 up at the tube cap, outside the PBS, and then centrifuged at 900 x g for 30 min. The dry gauze was
111 removed and the tube subject to a new centrifugation step (900 x g for 30 min) to obtain a pellet
112 which was resuspended in 1 ml PBS, transferred in a 1.5 ml sterile microtube and centrifuged at
113 17,500 x g for 15 min. The obtained pellet was first subjected to 3 freeze-thawing and then to DNA
114 extraction by NucleoSpin Tissue (Macherey-Nagel, Düren, Germany) following the manufacturer's
115 instruction (Galuppi et al., 2015). DNA samples were then subjected to nested-PCR, genotyping
116 and sub-typing methods, performed by PCR-RFLP analysis and sequencing as described by Caffara

117 et al. (2013). Briefly, the 18S rDNA gene was amplified by nested PCR with primers C1F-C1R and
118 C2F-C2R (Miller et al., 2006), following the PCR conditions reported by Xiao and Ryan (2008). PCR
119 amplicons were genotyped by PCR-RFLP analysis after digestion with *SspI* and *VspI* endonucleases
120 and restriction patterns were compared with the profiles reported by Xiao and Ryan (2008). The DNA
121 of positive samples was subtyped by nested PCR of the 60 KDa glycoprotein (gp60) gene, using
122 primers AL3532_f –AL3535_r (Alves et al., 2003) and AL3532_f-AL3534_r (Peng et al., 2001),
123 following the conditions reported by Xiao and Ryan (2008). For sequencing (18S rDNA and gp60),
124 the bands were excised, purified and sequenced with an ABI 3730 DNA analyzer at StarSEQ GmbH
125 (Mainz, Germany). The DNA trace files were assembled by Vector NTI Advance 11 software
126 (Invitrogen, Carlsbad,CA) and multiple sequence alignments were constructed using BioEdit.

127 The DNA of each sample was also subjected to qPCR assay, amplifying ~159 bp of the 18S rRNA
128 gene specific for *Cryptosporidium* spp. using JVAF (5'-ATGACGGGTAAACGGGGAAT-3'), JVAR
129 (5'- CCAATTACAAAACCAAAAA-3') primers and JVAP18S TaqMan probe (5'- Cy5-
130 CGCGCCTGCTGCCTTCCTTAGATG-BHQ-3') (Jothikumar et al., 2008). An Internal Positive
131 Control (TaqMan Exogenous Internal Positive Control Reagents) was included (IPC, Life
132 Technologies, Milan, Italy) in every reaction mixture (Malorny et al., 2004).

133 Briefly, all reactions consisted of 1X TaqMan® Fast Universal PCR Master Mix (2X) (Applied
134 Biosystems, Milan, Italy); 500 nM of each primer, 100 nM of the target probe, 2 µl of IPC Mix (10X),
135 0.4 µl of IPC DNA (50X) and 5 µl of DNA in 20 µl total volume. The cycling conditions were 95°C
136 for 2 min, followed by 45 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s and
137 extension at 72°C for 20 s. All DNA samples were tested in a Peltier based real-time PCR instrument
138 (Applied Biosystem 7500 Fast real-time PCR system, Life Technologies).

139 In order to establish the Limit Of Detection (LOD), the amplicons were cloned into a pEX-A plasmid
140 vector (Eurofins MWG Operon, Ebersberg, Germany). A standard curve based on Ct values obtained
141 from nine 10-fold serial dilutions of the plasmid in DNase- and RNase- free water were analyzed,

142 with an initial concentration of 5×10^8 plasmid copies/PCR. Three replicates of each dilution were
143 tested. Additionally, nine 2-fold serial dilutions of the plasmid were analyzed on a range of 400-0.78
144 gene copies/PCR. Six replicates of each dilution were tested. The LOD was identified as the lowest
145 concentration with all the six replicates positives.

146 Environmental samples were tested in duplicate. Each sample was considered positive with target Ct
147 values ≤ 36 for both replicates and negative with target Ct values > 36 and IAC Ct values < 40 for
148 one or both replicates.

149

150 Enumeration of *Cryptosporidium* spp. oocysts

151 A standard curve was used to estimate *Cryptosporidium* numbers in positive samples. To prepare the
152 standard curve, *Cryptosporidium* oocysts were purified from the faeces of an infected horse (*C.*
153 *parvum* IIdA23G1) using a salt flotation method (Elwin et al., 2001). Purified oocysts were
154 resuspended in deionized water, homogenized, and enumerated microscopically following staining
155 with a modified Ziehl-Neelson method. Dilutions were prepared containing approximately 50, 25,
156 12, 6, 3 and 1 oocyst in a total volume of 50 μ l and subjected to DNA extraction using QIAamp
157 DNA® Stool Mini Kit (Qiagen, Valencia, CA) and qPCR using the method described earlier.

158 The standard curve was linear in the range of 50 to 1 oocysts DNA/sample corresponding to a range
159 of 100 to 2 gene copies per PCR reaction, due to the presence of multiple 18S rRNA gene copies in
160 *Cryptosporidium* genome (Le Blancq et al., 1997), with R^2 of 0.98 (Fig 3).

161

162 **2.4 Statistical analyses**

163 Agreement between the two tests (nested-PCR and qPCR) was assessed by Cohen's Kappa statistic,
164 with K values of 0.00 to 0.20 indicating slight agreement, 0.21 to 0.40 indicating fair agreement, 0.41
165 to 0.60 indicating moderate agreement, 0.61 to 0.80 indicating substantial agreement and 0.81 to 1.00
166 indicating almost perfect agreement (Landis & Koch, 1977). McNemar's test was used to assess
167 significance of differences between the two tests, while a χ^2 test was performed to evaluate different

168 positive rates among sampling sites (floor vs wall) and among different materials covering the walls
169 (plaster, rubber or wood). Values of $p < 0.05$ were considered statistically significant.

170

171 **3. Results**

172

173 *Cryptosporidium* spp. was detected in 14 out of 175 environmental samples. Seven positive samples
174 were detected by both methods, 6 by qPCR only and 1 by nested-PCR only. The K Cohen test show
175 substantial agreement ($K = 0.65$; 95% CL) between the two tests. No statistical difference was
176 observed between the two tests (McNemar's $\chi^2 = 2.28$; $p = 0.065$).

177 In order to establish the Limit Of Detection (LOD) of the real-time PCR assay, a standard curve was
178 prepared from 10-fold serial dilutions of a plasmid containing the target 18S rDNA sequence. Ct
179 values showed a linear correlation (R^2 of 0.9989) with a linear range of detection from 8.7 to 0.7
180 \log_{10} gene copies/PCR (Fig 2). The PCR efficiency was 95.81%. Additionally 2-fold serial dilutions
181 of the plasmid were tested in six replicates per dilution in the range 400-0.78 gene copies/PCR. The
182 LOD, calculated as the lowest gene copy number for which all six PCR replicates were positive, was
183 1.56 and this corresponded to a mean Ct of 36.86 with a 95% CI of ± 0.58 (true mean range of 36.28
184 to 37.44) (Table 2).

185 The lowest dilution (1 oocyst/sample) had a mean Ct value of 34.21 and a 95% CI of ± 0.66 . The
186 range for the true mean was from 33.55 to 34.87 (Fig 3). The Ct values and the number of oocysts,
187 ranged from 29.67 to 36 and from < 1 to 11 respectively. Out of the 161 negative samples, 159 had
188 undetermined Ct value and two had Ct value > 36 . All the duplicate reactions had undetermined Ct
189 value. For the two samples with a Ct value > 36 , this value was obtained in only one of the duplicate
190 reactions. Both of these samples were negative by the nested PCR assay.

191 Eight out of 13 boxes (61.5%) had at least one sample that was positive for *Cryptosporidium*. Eleven
192 out of 95 (11.6%) samples from walls were positive, while only 2 out of 70 (2.8%) samples from
193 floor were positive. This difference was statistically significant ($\chi^2 = 4.22$; $p = 0.039$). Furthermore,

194 one (utility room A) out of two utility rooms was positive for *Cryptosporidium* spp. from the floor,
195 with the lowest Ct value and the highest number of oocysts.

196 Data from the eight boxes and utility room that had at least one *Cryptosporidium* positive sample
197 during the three foaling seasons are presented in Table 1.

198 No statistical differences were observed between the samples from plastered walls (7 positive out of
199 54, 13%) and rubber mats (3 out of 22, 13.6%) or wooden ones (1 out of 19, 5.3%).

200 PCR-RFLP analysis of the 18S rRNA gene allowed identification of *Cryptosporidium parvum* in 3
201 samples (box 2 in September and December 2011; box 9 in July 2013) and *Cryptosporidium* horse
202 genotype in 4 different samples (box 6, 7, 8 in July 2012 and box 8 in September 2012). All the
203 samples were successfully subtyped at the gp60 locus by sequencing ~800 bp of the PCR product.

204 Among *C. parvum*, 2 subtype families have been detected: IIaA15G2R1 and IIdA23G1, whereas
205 *Cryptosporidium* horse genotype belongs to subtype family VIa (VIaA15G4).

206

207 **4. Discussion**

208

209 *Cryptosporidium* spp. is a protozoan parasite of medical and veterinary significance and the
210 dissemination of resistant oocysts in the environment plays an important role in the epidemiology of
211 cryptosporidiosis (Fayer et al., 2008). In horses, cryptosporidiosis was first described in 1978 (Snyder
212 et al., 1978) and subsequently was reported in some surveys (Gajadhar et al., 1985; Xiao and Herd,
213 1994; Grinberg et al., 2003; Grinberg et al., 2009; Imhasly et al., 2009; Veronesi et al., 2010; Perrucci
214 et al., 2011; Caffara et al., 2013; Galuppi et al., 2015; Kostopoulou et al. 2015). The present research
215 focused on *Cryptosporidium* environmental contamination of the Equine Perinatology Unit EPU
216 where cryptosporidiosis in foals was already reported (Galuppi et al., 2015).

217 Out of the 175 samples tested, only 14 positives were identified. This result is one of the first
218 indications on the low level of *Cryptosporidium* contamination in equine perinatology Unit
219 environment; this could be due to the biosecurity measures applied to reduce the spread of infections

220 such as dedicated attire, disposable exam gloves and footbaths at every entry point to the stables, and
221 the cleaning of the boxes after every discharge/death.

222 In the boxes of this facility, a greater number of positive samples were obtained from walls than
223 floors, this could be due to the greater attention provided to cleaning of the floor of boxes, more
224 prominently contaminated by animal feces. In contrast, walls that probably are cleaned in a less
225 accurate way could represent a niche of fecal contamination.

226 No statistical differences were observed between the different wall material, even if some materials
227 can be more difficult to clean and favor the persistence of fecal debris.

228 Plastered walls are characterized by porosity, fragility and by the presence of bumps that make the
229 procedures of cleaning and disinfection more difficult, however the walls covered by rubber mats,
230 certainly easier to clean, were also positive. This could be explained by the fact that the latter were in
231 boxes housing critically ill foals often immunodeficient who shed high number of oocysts and are
232 frequently positive during the foaling seasons. The wooden walls are characterized by cracks,
233 representing a suitable place for *Cryptosporidium* oocysts. Atwill et al. (1998), in a study performed
234 for the evaluation of periparturient dairy cows and contact surfaces as a reservoir of *C. parvum*
235 infection in calves, detected *C. parvum* mainly in walls and floors of wooden calf hutches. In our
236 study, only one wooden wall sample was positive. It was collected in a box that housed a foal with
237 diarrhea, positive for *Cryptosporidium* spp. (personal communication), while the other samples from
238 wooden walls were from boxes housing asymptomatic animals, that excrete only minor number of
239 oocysts (Veronesi *et al.*, 2010; Perrucci *et al.*, 2011).

240 Regarding the comparison between nested-PCR and qPCR, both targeting the 18S rRNA gene for
241 *Cryptosporidium* spp., qPCR was able to detect the highest number of positive samples, however the
242 difference between the two tests was not statistically significant. De Waele et al. (2011) in a study
243 performed to compare four diagnostic tests (microscopic examination of smears stained with either
244 phenol-auramine, or fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium*

245 monoclonal antibody, nested-PCR and qPCR) for detection of *Cryptosporidium* oocysts, identifying
246 the 18S rRNA qPCR as the most sensitive and specific test.

247 Others have found that qPCR methods targeting the 18S rRNA gene perform better than those
248 targeting protein coding loci, including actin gene, for the detection of *C. parvum*/*C. hominis* in sheep
249 (Yang *et al.* 2009) and *C. parvum* in calves (Homem *et al.* 2012).

250 In our research all the samples negative by nested-PCR showed high Ct values (>33.68), which
251 equated to ≤ 1 oocyst. Yang *et al.* (2009) and Homem *et al.* (2012) similarly found that samples
252 negative by nested-PCR but positive by qPCR had high Ct values.

253 The qPCR protocol used in the present study allows the detection of DNA from less than 1 oocyst,
254 which is consistent with the 0.5 oocysts per reaction detection limit reported by Jothikumar *et al.*,
255 (2008). This can be explained by the presence of multiple 18S rRNA gene copies in *Cryptosporidium*
256 genome. Le Blance *et al.* (1997) showed that *C. parvum* has five 18S rRNA gene copies per sporozoite
257 genome, which, given that there are four sporozoites in an oocyst, translates to 20 18 S rRNA gene
258 copies per oocyst. A limitation of qPCR assays targeting the 18S rRNA gene is that they are only
259 genus specific. Species specific qPCR assays have been described, but these target single copy genes
260 and therefore have reduced sensitivity compared to assays targeting the 18S rRNA gene (Fontaine
261 and Guillot, 2002; Guy *et al.*, 2003; De Waele *et al.*, 2012). Moreover a study aimed to determine the
262 applicability of ten TaqMan-based qPCR assay for detecting *C. hominis*, *C. parvum* or
263 *Cryptosporidium* spp. oocysts in water matrix, revealed that genus-qPCR target 18S rRNA can detect
264 the lowest number of oocysts (Staggs *et al.*, 2013). Therefore, even if it is not possible to define the
265 species, the use of *Cryptosporidium* spp. qPCR targeting the 18S rRNA gene allows to detect a very
266 low number of oocysts in environmental samples where the parasite load is generally low. Moreover,
267 this gene contains polymorphic regions permitting, by sequencing, the identification of new species
268 or genotypes that can cause disease in humans, like the recent identification of *C. cuniculus* or horse
269 and skunk genotypes (Robinson *et al.*, 2008; Chalmers *et al.*, 2011).

270 All the positive samples in nested PCR were confirmed by qPCR, except one.

271 Although nested-PCR was less sensitive than qPCR, RFLP analysis of the 18S rRNA nested-PCR
272 amplicons allowed isolates to be identified to the species level and the gp60 sequencing permitted the
273 identification of the samples under study as *C. parvum* with two different gp60 subtypes
274 (IIaA15G2R1 and IIdA23G1) and *Cryptosporidium* horse genotype (gp60 subtype VIaA15G4).
275 The subtypes detected on the surfaces are the same affecting hospitalized animals during the
276 considered time period. *C. parvum* IIaA15G2R1, which was detected in the environment at the end
277 of the 2011 foaling seasons, was also detected in foals housed at the facility during the same period
278 (Diaz et al., 2012). Similarly, the horse genotype was detected in the environment and in foals during
279 the 2012 foaling season (Caffara et al., 2013). *C. parvum* was the only species detected in the
280 environment in 2013 and was also diagnosed in two foals and seven humans of the same facility
281 during the same period (Galuppi et al., 2016)

282 Even if some authors reported that in humans the mean infectious dose for some isolate of *C. parvum*
283 can be as low as 12 or as high as 2066 oocysts (Messner et al., 2001; Okhuysen and Chappel, 2002),
284 Pereira et al. (2002) reported that a single oocyst is sufficient to cause infection and disease in an
285 animal model. In this study, *Cryptosporidium* DNA of less than 1 oocyst was detected, testing only a
286 small area of the floor or wall, suggesting that probably a higher number of oocysts was present in
287 the entire box.

288 Even if the disinfectants used in the EPU have not always proved to be effective against
289 *Cryptosporidium* oocysts (Ares-Mazas et al., 1997; Weir et al., 2002), quantification of oocysts by
290 qPCR showed a probable reduction or negativity of parasite load of the environmental surfaces after
291 washing and disinfecting procedure as observed in box 2 in 2011, in boxes 6, 8, 9, 13 in 2012 and in
292 box 9 in 2013 (see table 1).

293 In 2013 boxes 1, 2 and 11 were negative in July, the end of foaling season, but were positive, even if
294 at very low amount (Ct 36 and Ct 34.15 from floor and wall of box 1; Ct 36 from wall of box 2; Ct
295 35.9 from floor of box 11, with < 1 oocyst for all samples) in September after the washing procedures.
296 We could hypotized that the negativity observed in box 1 and 11 in July 2013 could be due to the

297 deep disinfection procedures applied in these boxes after the death/discharge of two foals, positive
298 for *Cryptosporidium* (Galuppi et al., 2016), allowing a decrease of parasitic load in the environment.
299 Since the variability of the results due to the randomness of sampling is a well-known event when the
300 contamination is low and thus not uniformly distributed on the surfaces, the low amount of oocysts
301 detected in December 2013 could be explained by the presence of minimal faeces residual. Even if
302 none of the positive foals has been hosted in box 2, the very low positivity observed could be due to
303 its close proximity to the utility room, where the highest oocysts amount has been detected.
304 The high number of oocysts found in the utility room at the entrance of box 1, the highest among all
305 samples, highlighted the importance of disinfection procedures that have to be performed with
306 attention in all the areas of EPU that could represent a source of infection.

307

308 **5. Conclusion**

309 In the present study, *Cryptosporidium* DNA was detected on environmental surfaces of an Equine
310 Perinatology Unit, suggesting that EPU could represent a potential reservoir of *Cryptosporidium*
311 oocysts that might be transmitted to humans and animals. Due to the low number of oocysts detected
312 in the environment, qPCR should be selected as a highly sensitive molecular technique. In order to
313 reduce the risk of transmission, more efficient cleaning and disinfecting procedures should be
314 considered and extended to all rooms of the EPU also those not directly hosting animals.

315

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320

321

322 **References**

- 323 Alves, M., Xiao, L., Sulaiman, I., Lal, A.A., Matos, O., Antunes, F., 2003. Subgenotype analysis of
324 *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. *J. Clin.*
325 *Microbiol.* 41, 2744-2747.
- 326 Anderson, B.C., Donndelinger, T, Wilkins, R., Smith J., 1982. Cryptosporidiosis in a veterinary
327 student. *J. Am. Vet. Med. Assoc.* 180, 408-409.
- 328 Atwill, E.R., Harp, J.A., Jones, T., Jardon, P.W., Checel, S., Zylstra, M., 1998. Evaluation of
329 periparturient dairy cows and contact surfaces as a reservoir of *Cryptosporidium parvum* for
330 calfhoo infection. *Am. J. Vet. Res.* 59, 1116-21.
- 331 Caffara, M., Piva, S., Pallaver, F., Iacono, E., Galuppi, R., 2013. Molecular characterization of
332 *Cryptosporidium* spp. from foals in Italy. *Vet. J.* 198, 531-533.
- 333 Castro-Hermida, J.A, Garcia-Preedo, I., Almeida, A., Gonzalez-Werleta, M., Da Costa, J.M.C.,
334 Mezo, M., 2009. Detection of *Cryptosporidium* spp. and *Giardia duodenalis* in surface
335 water: a health risk for humans and animals. *Water. Res.* 43, 41Chalmers, R.M., Elwin, K.,
- 336 Hadfield, S.J., Robinson, G., 2011. Sporadic human cryptosporidiosis caused by *Cryptosporidium*
337 *cuniculus*, United Kingdom, 2007-2008. *Emerg. Infect. Dis.* 17, 536-538.
- 338 Chalmers, R.M., Katzer, F., 2013. Looking for *Cryptosporidium*: the application of advances in
339 detection and diagnosis. *Trends Parasitol.* 29, 237-251.
- 340 De Waele, V., Berzano, M., Berkvens, D., Speybroeck, N., Lowery, C., Mulcahy, G.M., Murphy,
341 T.M., 2011. Age-stratified Bayesian analysis to estimate sensitivity and specificity of four
342 diagnostic tests for detection of *Cryptosporidium* oocysts in neonatal calves. *J. Clin.*
343 *Microbiol.* 49, 76-84.
- 344 De Waele, V., Berzano, M., Speybroeck, N., Berkvens, D., Mulcahy, G.M., Murphy, T.M., 2012.
345 Peri-parturient rise of *Cryptosporidium* oocysts in cows: new insights provided by duplex
346 quantitative real-time PCR. *Vet. Parasitol.* 189, 366-368.

347 Diaz, P., Castagnetti, C., Marchesi, B., Soilan, M., Lopez, C.M., Diez-Banos, P., Morrondo, P.,
348 Poglayen, G., 2012. Investigation of the zoonotic potential of *Cryptosporidium* in a
349 diarrhoeic foal, p 251. In *Mappe parassitologiche XXVII Congresso Nazionale. Società*
350 *Italiana di Parassitologia. Alghero, 26-29 giugno, vol18, p.251.*

351 Elwin, K., Chalmers, R.M., Roberts, R., Guy, E.C., Casemore, D.P., 2001. Modification of a rapid
352 method for the identification of gene-specific polymorphisms in *Cryptosporidium parvum*
353 and its application to clinical and epidemiological investigations. *Appl. Environ. Microbiol.*
354 *67, 5581-5584.*

355 Fayer, R., 2004. *Cryptosporidium: a water-borne zoonotic parasite. Vet. Parasitol.* 126, 37-56.

356 Fayer, R., 2008. General Biology p 1-35. In Fayer R, Xiao L (ed), *Cryptosporidium and*
357 *cryptosporidiosis. 2th ed. CRC Press of Taylor and Francis Group, NW.*

358 Feng, Y., Zhao, X., Chen, J., Jin, W., Zhou, X., Li, N., Wang, L., Xiao, L., 2011. Occurrence,
359 source, and human infection potential of *Cryptosporidium* and *Giardia* spp. in source and tap
360 water in Shanghai, China. *Appl. Environ. Microbiol.* 77, 3609-3616.

361 Fontaine, M., Guillot, E., 2002. Development of a TaqMan quantitative PCR assay specific for
362 *Cryptosporidium parvum. FEMS Microbiol. Lett.* 27, 13-17.

363 Gait, R., Soutar, R.H., Hanson, M., Fraser, C., Chalmers, R., 2008. Outbreak of cryptosporidiosis
364 among veterinary students. *Vet. Rec.* 162, 843-845.

365 Gajadhar, A.A., Caron, J.P., Allen, J.R., 1985. Cryptosporidiosis in two foals. *Can. Vet. J.* 26, 132-
366 134.

367 Galuppi, R., Piva, S., Castagnetti, C., Iacono, E., Tanel, S., Pallaver, F., Fioravanti, M.L., Zanoni,
368 R.G., Tampieri, M.P., Caffara, M., 2015. Epidemiological survey on *Cryptosporidium* in an
369 Equine Perinatology Unit. *Vet. Parasitol.* 210, 10-18.

370 Galuppi, R., Piva, S., Castagnetti, C., Sarli, G., Iacono, E., Fioravanti, M.L., Caffara, M., 2016.
371 *Cryptosporidium parvum: From foal to veterinary students. Vet. Parasitol.* 219, 53-56.

372 Grinberg, A., Oliver, L., Learmonth, J.J., Leyland, M., Roe, W., Pomroy, W.E., 2003. Identification
373 of *Cryptosporidium parvum* 'cattle' genotype from a severe outbreak of neonatal foal
374 diarrhoea. *Vet. Rec.* 153, 628-631.

375 Grinberg, A., Pomroy, W.E., Carslak, H.B., Shi, Y., Gibson, I.R., Drayton, B.M., 2009. A study of
376 neonatal cryptosporidiosis of foals in New Zealand. *N. Z. Vet. J.* 57, 284-289.

377 Guy, R.A., Payment, P., Krull, U.J., Horgen, P.A., 2003. Real-time PCR for quantification of
378 *Giardia* and *Cryptosporidium* in environmental water samples and sewage. *Appl. Environ.*
379 *Microbiol.* 69, 5178-5185.

380 Haugland, R.A., Siefring, S.C., Wymer, L.J., Brenner, K.P., Dufour, A.P., 2005. Comparison of
381 *Enterococcus* measurements in freshwater at two recreational beaches by quantitative
382 polymerase chain reaction and membrane filter culture analysis. *Water Res.* 39, 559-568.

383 Henriksen, S.A., Pohlenz, J.F., 1981. Staining of cryptosporidia by a modified Ziehl-Neelsen
384 technique. *Acta Vet. Scand.* 22, 628-631.

385 Homem, C.G., Nakamura, A.A., Silva, D.C., Teixeira, W.F., Coelho, W.M., Meireles, M.V., 2012.
386 Real-time PCR assay targeting the actin gene for the detection of *Cryptosporidium parvum*
387 in calf fecal samples. *Parasitol. Res.* 110, 1741-1745.

388 Hong, S., Kim, K., Yoon, S., Park, W.Y., Sim, S., Yu, J.R., 2014. Detection of *Cryptosporidium*
389 *parvum* in environmental soil and vegetables. *J. Korean Med. Sci.* 29, 1367-1371.

390 Imhasly, A., Frey, C.F., Mathis, A., Straub, R., Gerber, V., 2009. [Cryptosporidiose (*C. parvum*) in
391 a foal with diarrhea]. *Schweiz. Arch. Tierheilkd.* 151, 21-26.

392 Jothikumar, N., Da Silva, A.J., Moura, I., Qvarnstrom, Y., Hill, V.R., 2008. Detection and
393 differentiation of *Cryptosporidium hominis* and *Cryptosporidium parvum* by dual TaqMan
394 assays. *J. Med. Microbiol.* 57, 1099-1105.

395 Khaldi, S., Ratajezak, M., Gargala, G., Fournier, M., Berthe, T., Favennec, L., Dupont, J.P., 2011.
396 Intensive exploitation of a karst aquifer leads to *Cryptosporidium* water supply
397 contamination. *Water Res.* 45, 2906-2914.

398 Kinross, P, Beser, J, Troell, K., Silverlås, C., Björkman, C., Lebbad, M., Winięcka-Krusnell, J.,
399 Lindh, J., Löfdahl, M., 2015. *Cryptosporidium parvum* infections in a cohort of veterinary
400 students in Sweden. *Epidemiol. Infect.* 143, 2748-2756.

401 Konkle, D.M., Nelson, K.M., Lunn, D.P., 1997. Nosocomial transmission of *Cryptosporidium* in a
402 veterinary hospital. *J. Vet. Intern. Med.* 11, 340-343.

403 Kostopoulou, D., Casaert, S., Tzanidakis, N., van Doorn, D., Demeler, J., von Samson-
404 Himmelstjerna G., Saratsis, A., Voutzourakis, N., Ehsan, A., Doornaert, T., Looijen, M., De
405 Wilde, N., Sotiraki, S., Claerebout, E., Geurden, T., 2015. The occurrence and genetic
406 characterization of *Cryptosporidium* and *Giardia* species in foals in Belgium, The
407 Netherlands, Germany and Greece. *Vet. Parasitol.* 211, 170-174.

408 Landis, J.R., Koch, G.G., 1977. The Measurement of Observer Agreement for Categorical Data.
409 *Biometrics* 33, 159-174

410 Le Blancq, S.M., Khramtsov, N.V., Zamani, F., Upton, S.J., Wu, T.W., 1997. Ribosomal RNA gene
411 organization in *Cryptosporidium parvum*. *Mol. Biochem. Parasit.* 90, 463-478.

412 Levine, J.F., Levy, M.G., Walker, R.L., Crittenden, S., 1988. Cryptosporidiosis in veterinary
413 students. *J. Am. Vet. Med. Assoc.* 193, 1413-1414.

414 Maida, C., Di Benedetto, M.A., Firenze, A., Calamusa, G., Di Piazza, F., Milici, M., Romano, N.,
415 2008. Surveillance of the sanitary conditions of a public swimming pool in the city of
416 Palermo (Italy). *Ig. Sanità. Pubbl.* 64, 581-593.

417 Malorny, B., Paccassani, E., Fach, P., Bunge, C., Martin, A., Helmuth, R., 2004. Diagnostic real-
418 time PCR for detection of *Salmonella* in food. *Appl. Environ. Microbiol.* 70, 7046-7052.

419 Masago, Y., Oguma, K., Katayama, H., Ohgaki, S., 2006. Quantification and genotyping of
420 *Cryptosporidium* spp. in river water by quenching probe PCR and denaturing gradient gel
421 electrophoresis. *Water Sci. Technol.* 54, 119-126.

422 Messner, M.J., Chappell, C.L., Okhuysen, P.C., 2001. Risk assessment for *Cryptosporidium*: a
423 hierarchical Bayesian analysis of human dose response data. *Water Res.* 35, 3934-3940.

424 Miller, W.A., Gardner, I.A., Atwill, E.R., Leutenegger, C.M., Miller, M.A., Hedrick, R.P., Melli,
425 A.C., Barnes, N.M., Conrad, P.A., 2006. Evaluation of methods for improved detection of
426 *Cryptosporidium* spp. in mussels (*Mytilus californianus*). J. Microbiol. Methods 65, 367-
427 379.

428

429 Nolan, M.J., Jex, A.R., Koehler, A.V., Haydon, S.R., Stevens, M.A., Gasser, R.B., 2013.
430 Molecular-based investigation of *Cryptosporidium* and *Giardia* from animals in water
431 catchments in southeastern Australia. Water Res. 47, 1726-1740.

432 Okhuysen, P.C., Chappell, C.L., 2002. *Cryptosporidium* virulence determinants--are we there yet?
433 Int. J. Parasitol. 32, 517-525.

434 Peng, M.M., Matos, O., Gatei, W., Das, P., Stantic-Pavlinic, M., Bern, C., Sulaiman, I.M.,
435 Glaberman, S., Lal, A.A., Xiao, L., 2001. A comparison of *Cryptosporidium* subgenotypes
436 from several geographic regions. J. Eukaryot. Microbiol. (Suppl. s1), 28S-31S.

437 Pereira, C., Atwill, E.R., Barbosa, A.P., Silva, S.A.E., Garcia-Zapata, M.T.A., 2002. Intra-familial
438 and extra-familial risk factors associated with *Cryptosporidium parvum* infection among
439 children hospitalized for diarrhea in Goiânia, Goiás, Brazil. Am. J. Trop. Med. Hyg. 66,
440 787-793.

441 Perrucci, S., Buggiani, C., Sgorbini, M., Cerchiai, I., Otranto, D., Traversa, D., 2011.
442 *Cryptosporidium parvum* infection in a mare and her foal with foal heat diarrhoea. Vet.
443 Parasitol. 182, 333-336.

444 Pohjola, S., Oksanen, H., Jokipii, L., Jokipii, A.M.M., 1986. Outbreak of cryptosporidiosis among
445 veterinary students. Scand. J. Infect. Dis. 18, 173-178.

446 Preiser, G., Preiser, L., Madeo, L., 2003. An Outbreak of Cryptosporidiosis Among Veterinary
447 Science Students Who Work With Calves. J. Am. Coll. Health 51, 213-215.

448 Reif, J.S., Wimmer, L., Smith, J.A., Dargatz, D.A., Cheney, J.M., 1989. Human cryptosporidiosis
449 associated with an epizootic in calves. Am. J. Public Health 79, 1528-1530.

450 Robinson, G., Elwin, K., Chalmers, R.M., 2008. Unusual *Cryptosporidium* genotypes in human
451 cases of diarrhea. *Emerg. Infect. Dis.* 14, 1800-1802.

452 Santin, M., 2013. Clinical and subclinical infections with *Cryptosporidium* in animals. *N. Z. Vet. J.*
453 61, 1-10.

454 Smith, H.N., Nichols, R.A., 2010. *Cryptosporidium*: detection in water and food. *Exp. Parasitol.*
455 124, 61-79.

456 Snyder, S.P., England, J.J., McChesney, A.E., 1978. Cryptosporidiosis in immunodeficient Arabian
457 foals. *Vet. Pathol.* 15, 12-17.

458 Staggs, S.E., Beckman, E.M., Keely, S.P., Mackwan, R., Ware, M.W., Moyer, A.P., Ferretti, J.A.,
459 Sayed, A., Xiao, L., Villegas, E.N., 2013. The Applicability of TaqMan-Based Quantitative
460 Real-Time PCR Assays for Detecting and Enumerating *Cryptosporidium* spp. Oocysts in the
461 Environment. *PLoS One* 21, e66562.

462 Veronesi, F., Passamonti, F., Cacciò, S., Diaferia, M., Piergili Fioretti, D., 2010. Epidemiological
463 survey on equine *cryptosporidium* and *giardia* infections in Italy and molecular
464 characterization of isolates. *Zoonoses Public. Health* 57, 510-517.

465 Xiao, L., Herd, R.P., 1994. Epidemiology of equine *Cryptosporidium* and *Giardia* infections.
466 *Equine Vet. J.* 26, 14-17.

467 Xiao, L., Ryan, U.M., 2008. Molecular epidemiology. In: Fayer, R., Xiao, L. (Eds.).
468 *Cryptosporidium* and Cryptosporidiosis. CRC Press/IWA Publishing, Boca Raton, USA,
469 pp.119-171.

470 Yang, R., Jacobson, C., Gordon, C., Ryan, U., 2009. Prevalence and molecular characterisation of
471 *Cryptosporidium* and *Giardia* species in pre-weaned sheep in Australia. *Vet. Parasitol.*
472 161,19-24.

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477 **Figure Caption**

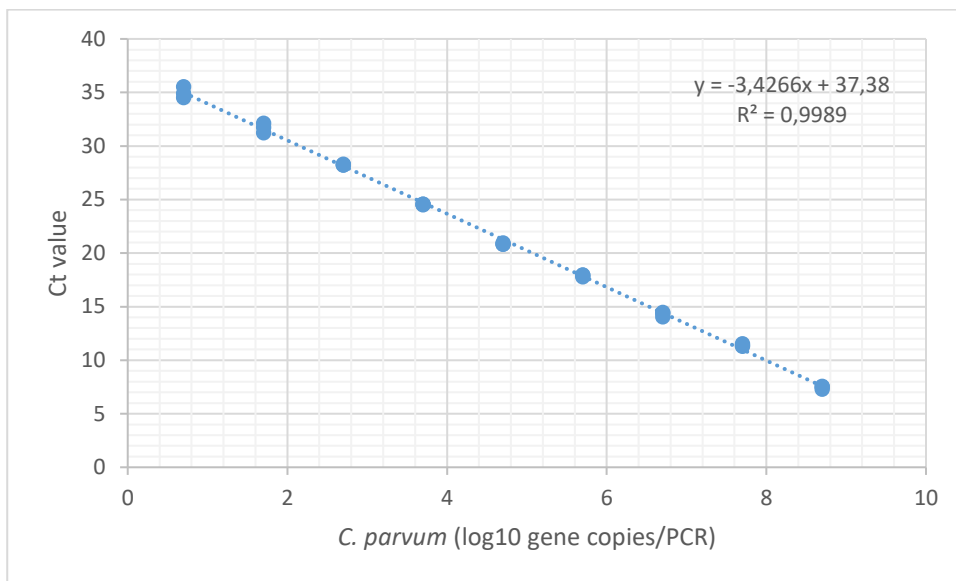
478 Fig.1: Planimetry of EPU

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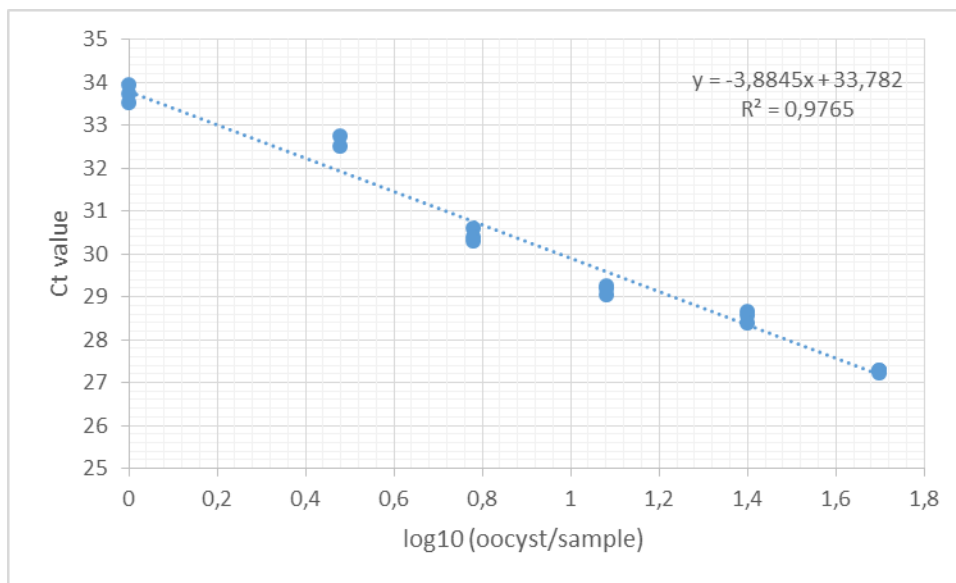
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Fig 2: Standard curve based on Ct values obtained from nine 10-fold serially diluted *C. parvum* 18S rDNA target gene cloned in pEX-A plasmid vector starting from an initial concentration of 5×10^8 gene copies/PCR.



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Fig 3: Standard curve based on Ct values of 50, 25, 12, 6, 3, 1 oocyst/sample (corresponding to a range of 100-2 gene copies/PCR).

