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Observational Study on Cryptosporidiosis in an Equine Perinatology Unit

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Lanci, A., Mariella, J., Iacono, E., Caffara, M., Piva, S., Galuppi, R., et al. (2018). Observational Study on Cryptosporidiosis in an Equine Perinatology Unit. JOURNAL OF EQUINE VETERINARY SCIENCE, 71, 51-56 [10.1016/j.jevs.2018.09.009].

Availability:

This version is available at: <https://hdl.handle.net/11585/649004> since: 2019-07-15

Published:

DOI: <http://doi.org/10.1016/j.jevs.2018.09.009>

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The final published version is available online at: <https://doi.org/10.1016/j.jevs.2018.09.009>

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16 **Observational Study on Cryptosporidiosis in an Equine Perinatology Unit**

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25

26 **ABSTRACT**

27 The present study aimed to describe clinical signs of cryptosporidiosis in neonatal foals hospitalized
28 in an Equine Perinatology Unit (EPU) and to compare the clinical signs between *Cryptosporidium*
29 *parvum* and *Cryptosporidium* horse genotype infection. The study was divided into 2 parts. In the
30 retrospective study, 9 foals infected by *C. parvum* were considered. In the prospective study, 70 foals,
31 less than 15 days old, were prospectively included. Historical and clinical data were recorded and, in
32 the prospective study, multiple fecal samples were collected. *Cryptosporidium parvum* (n =13) and
33 *Cryptosporidium* horse genotype (n = 7) were isolated. In 4 foals, there was a mixed infection with
34 both the *Cryptosporidium*. Diarrhea, when present, showed similar duration and characteristics.
35 Sixteen foals showed decreased abdominal sounds and colic pain before evidence of diarrhea.
36 Nineteen foals had hyperthermia at least once. Although survival rates were similar between *C.*
37 *parvum* (77%), *C.* horse genotype (100%) and cryptosporidial mixed infection (100%), foals affected
38 by *C. parvum* presented anorexia (P <0.0031) and received specific therapy (P <0.014) more
39 frequently than the others. Recorded data strengthen the think that *C. parvum* infection is more severe
40 in foals, suggesting that they would have developed host adaptations in response to the *C.* horse
41 genotype or that *C. parvum* is a more pathogenic strain. Since healthy and asymptomatic foals can
42 shed oocysts of *Cryptosporidium* spp., students and staff should always wear the personal protective
43 equipment to avoid zoonotic infection.

44

45 **Keywords:** foal, *Cryptosporidium*, diarrhea, zoonosis.

46 INTRODUCTION

47 Diarrhea is a very common clinical sign in newborn foals and could be a sign of infectious diseases,
48 hypoxic gut injury, nutritional upsets or changes in intestinal flora, that quickly give rise to systemic
49 manifestations [1,2]. The most common causes of diarrhea in newborn foals are: Rotavirus,
50 *Clostridium perfringens* and *difficile*, *Salmonella* spp and *Cryptosporidium* [3].

51 Among parasites, *Cryptosporidium* was described for the first time in the 1978 by Snyder et al. [4] in
52 5 immunodeficient Arabian foals with diarrhea. The authors thought that this parasite only affected
53 immunocompromised foals, but subsequently it has been associated with sporadic and/or outbreak of
54 diarrhea even in immunocompetent horse [5,6].

55 Because of the high economic losses, due to growth retardation determined by diarrhea, much more
56 has been written about cryptosporidiosis in calves, lambs and kids [7,8]. In these animals,
57 cryptosporidiosis, mainly due to *Cryptosporidium parvum*, has been described as severe disease,
58 characterized by yellow, malodorous feces, with consistency from soft to liquid, associated with
59 depression, abdominal pain and anorexia [8-10].

60 In horse, until 2003 only *Cryptosporidium parvum* was known [11-14]. In 2003, *Cryptosporidium*
61 horse genotype was described for the first time in Przewalski adult horse [15], and subsequently also
62 isolated in healthy foals, less than one month old, in the New York State [16].

63 Epidemiological studies about cryptosporidial infection in horses were conducted in Louisiana [17],
64 United Kingdom [18], New Zealand [13], New York State [16], Trinidad [19], Sao Paulo State [20],
65 Algeria [21], Kentucky [22], Belgium, The Netherlands, Germany and Greece [23], China [24] and
66 Brazil [25]. In particular, studies conducted in different farms of Central Italy revealed a great
67 variability of prevalence, from 0% to 31.25% [6,14,26].

68 Many papers reported the prevalence of *Cryptosporidium* spp. in faeces of healthy adult horses and
69 foals [12,16, 20,21,23,27,28], but clinical signs and risk factors are still unclear [14,29].

70 In the framework of a project about cryptosporidiosis in foals hospitalized at the Equine Perinatology
71 Unit “Stefano Belluzzi” (EPU), of the Department of Veterinary Medical Sciences, *Alma Mater*

72 *Studiorum* – University of Bologna, where *Cryptosporidium parvum* e *Cryptosporidium* horse
73 genotype were isolated [26,30,31], the present study aimed to describe the clinical signs of
74 cryptosporidiosis.

75

76 **MATERIALS AND METHODS**

77 **Experimental design**

78 The present study was divided into 2 parts:

79 *Retrospective study* - 6 foals (5 Italian Trotter and 1 Saddlebred) from foaling season 2007 and 3 foals
80 (2 Italian Trotter and 1 Quarter horse) from foaling season 2011 were included. All these animals,
81 hospitalized at the EPU, showed diarrhea and a fecal sample positive for *Cryptosporidium parvum*
82 by both modified Ziehl–Neelsen [32] and PCR [26].

83

84 *Prospective study* (foaling seasons 2012 and 2013) – all foals, less than 15 days old, hospitalized at
85 EPU during the foaling seasons 2012 and 2013 with or without diarrhea, were prospectively included.

86

87 **Data collection**

88 All the foals were subjected to a complete clinical examination at admission, and the following data
89 were recorded: breed, sex, gestational age (days), age at admission (hours), blood culture result, serum
90 IgG concentration (mg/dL), neutrophil blood count, and diagnosis. They were classified on the basis
91 of the most life-threatening condition at admission following the guidelines reported by Castagnetti
92 et al. (2010) [33]. Failure of passive transfer of immunity (FPT) was defined when, at more than 18
93 hours of life, serum IgG concentration was <800 mg/dL. During the hospitalization, foals were
94 clinically evaluated at least twice a day and the following data were recorded: rectal temperature, age
95 and days of hospitalization at the onset of symptoms, gastrointestinal symptoms such as anorexia,
96 diarrhea (presence, type and duration), colic pain, characteristics of abdominal sounds (normal,
97 increased, decreased, or absent), necessity of treatment due to cryptosporidiosis and type of treatment

98 (adsorbents, lactic ferments, antibiotics, antidiarrheal drugs, analgesic drugs, intravenous fluids and
99 plasma, total parenteral nutrition - TPN), hospitalization length and outcome. Finally, whether foals
100 with cryptosporidiosis survived, died spontaneously, or were euthanized was recorded.
101 Foals with diarrhea were isolated inside the unit, applying a specific internal isolation protocol. All
102 the operators were trained to apply the correct isolation practices with infectious animals (personal
103 protective equipment - PPE: disposables gloves, boot covers and plastic smocks) and the boxes were
104 always cleaned with specific and dedicated shovel, broom and barrow.

105

106 **Stool sample collection**

107 During the foaling seasons 2007 and 2011, fecal samples were collected only in case of diarrhea,
108 while during the foaling seasons 2012 and 2013, the protocol reported by Galuppi et al. (2015) [26]
109 was applied as follow: in foals born at EPU, fecal samples were collected 4 days after birth and then
110 every 2 days until discharge; in foals hospitalized after birth (with or without diarrhea), fecal samples
111 were collected at admission, 4 days later and then every 2 days until discharge. In all animals,
112 sampling was made directly from rectal ampulla to avoid environmental contamination. Immediately
113 after collection, each sample was identified through animal's name, date, progressive number and it
114 was kept refrigerated (+4°C) until processing. If the foal had not expelled meconium at admission,
115 sampling was performed at the onset of the first milk feces.

116 All the fecal samples were subjected to bacteriological culture (*E. coli*, *Clostridium* spp, *Salmonella*
117 spp), Rotavirus and *Cryptosporidium* analysis.

118 Each fecal sample was subjected to microscopic and molecular analysis for *Cryptosporidium* spp. as
119 previously described [26,30]. Each stool specimen was homogenized in distilled water, filtered
120 through gauze and centrifuged at $900 \times g$ for 30 min, the sediment was in part streaked onto a slide,
121 stained with modified Ziehl–Neelsen (Henriksen and Pohlens, 1981) method and observed under a
122 light microscope (400× and 1000×magnification), in part frozen at –20°C for molecular analysis. The
123 DNA was extracted by QIAamp DNA Stool Mini Kit (Qiagen), amplified by nested PCR of the 18S

124 rRNA. The positive samples were genotyped by restriction fragment length polymorphism (RFLP)
125 analysis and subtyped by nested PCR of the 60 kDa glycoprotein (gp60) gene following the conditions
126 of [34].

127

128 **Statistical analysis**

129 Data were analyzed for normality with the Kolmogorov–Smirnov test. Given the non-normal
130 distribution of data, nonparametric tests were used for statistical analysis.

131 Fisher’s exact test was used to test the differences between the categorical variables. The differences
132 in proportion between diarrheic foals positive to *Cryptosporidium* spp and negative diarrhoeic foals
133 were tested for: sex, age (greater or less than 72 hours of life), anorexia, prematurity, perinatal
134 asphyxia syndrome, FPT, sepsis and exitus, The differences between foals affected by two genotypes
135 of *Cryptosporidium* were tested for: sex, anorexia, necessity of treatment, characteristics of
136 abdominal sounds, hypertermia, FPT and exitus. Kruskal -Wallis test and Student t test were
137 performed to determine the differences between numeric variables (age at admission, gestational age,
138 neutrophil count, duration of diarrhea and hospitalization length) between foals with and without
139 diarrhea and between foals affected by two genotypes of *Cryptosporidium*. Summary data were
140 reported as median \pm standard error (SE). All analyses were performed with a commercial software
141 (Analyse-it Software Ltd., Leeds, West Yorkshire, England). Values of $P < 0.05$ were considered
142 significant.

143

144 **RESULTS**

145 *Retrospective study*

146 In the 2007 foaling season, the onset of clinical signs of cryptosporidiosis in the 6 included foals
147 occurred at 6 ± 1.6 days of life (range 1.5-14) and 5.7 ± 1.7 days of hospitalization (range 0-14; 3 foals
148 were born at EPU). None foal died or was euthanized and the median hospitalization length was 35
149 ± 5.1 days (range 17-45).

150 During the 2011 foaling season, none of the three enrolled foals was born at EPU. The onset of clinical
151 signs of cryptosporidiosis occurred at 9 ± 1.9 days of life (range 5-13) and 7 ± 2.1 days of
152 hospitalization (range 3.5-12.5). One foal (1/3 foals, 33.3%) was discharged after 40 days of
153 hospitalization. One foal (1/3 foals, 33.3%) was euthanized with the owner's consent after 27 days
154 due to financial constraints and one foal (1/3 foals, 33.3%) after 20 days due to worsening health
155 conditions.

156 Clinical data collected at admission (sex, age at admission, gestational age, FPT, neutrophil count
157 and major diagnosis) are shown in Table 1 while symptoms and outcome are listed in Table 2.

158

159 *Prospective study*

160 During the 2012 and 2013 foaling seasons, 70 foals were hospitalized at EPU. Twelve/70 foals were
161 excluded from the statistical analysis: in 11/12 (91.7%) foals the sampling protocol was not complete,
162 since they were euthanized or died within 24 hours of hospitalization without showing clinical signs
163 of Cryptosporidiosis; 1/11 (9.1%) foal was excluded because affected by a mixed infection (Rotavirus
164 and both *Cryptosporidium* genotypes). Therefore, 58 foals were considered in the study (22 Italian
165 Trotters, 19 Saddlebreds, 9 Arabian Horses, 5 Quarter Horses, 1 Paint Horse, 1 Missouri Fox Trotter
166 and 1 pony Shetland).

167 Eighteen/58 (31.2%) foals were born at EPU and 40/58 (68.9%) were referred. Seven foals were
168 euthanized for poor clinical condition: 1/7 (14.3%) foal was positive to *C. parvum*, while none of the
169 euthanized foals was positive for *Cryptosporidium* spp.

170 Clinical data collected at admission are shown in Table 1; symptoms and outcome are listed in Table
171 2.

172 The onset of clinical signs in positive foals started at 5 ± 0.9 days of life (range 1-13), after 2.5 ± 0.7
173 days of hospitalization (range 0-6.5). Fifteen out of 58 animals (25.8%) tested positive for
174 *Cryptosporidium* spp.: 4/15 (26.7%) for *C. parvum* (2 in 2012 and 2 in 2013), 7/15 (46.7%) for
175 *Cryptosporidium* horse genotype (in 2012) and 4/15 (26.7%) had mixed infection with both

176 *Cryptosporidium* spp (in 2012). In foals positive for *C. parvum*, the duration of hypomotility was 1
177 ± 0.1 days (range 0.5-2). In foals with cryptosporidial mixed infection, the duration of hypomotility
178 was 1.5 ± 0.1 days (range 1-1.5).

179 No significant differences were found in clinical data collected at admission between foals negative
180 and positive for *Cryptosporidium* spp. ($P > 0.05$), using Student's t test and Fisher's Exact test.

181 All the 24 positive foals showed gastrointestinal symptoms, except one completely asymptomatic
182 foal (4.2%) positive for *Cryptosporidium* horse genotype. Diarrhea, when present, showed similar
183 characteristics in both *Cryptosporidium* infections: watery, yellowish and foul smelling. In 16/24
184 (66.7%) foals, dehydration, anorexia and loss of electrolyte lead to the necessity of intensive therapy
185 with intravenous fluids, plasma and TPN. During diarrhea episodes, all the foals showed increased
186 abdominal sounds. In 16/24 (66.7%) foals, before evidence of diarrhea, the auscultation of the
187 abdomen revealed decreased abdominal sounds. Colic pain symptoms as rolling, bruxism, abrade or
188 get in decubitus continuously was observed in 16/24 (66.7%) foals. In 11/16 (68.7%) all the
189 mentioned above symptoms were present.

190 Although survival rates were not significantly different between the three groups (*C. parvum* 77%,
191 *Cryptosporidium* horse genotype 100% and cryptosporidial mixed infection 100%), Fisher's exact
192 test revealed that foals affected by *C. parvum* have more probability to present anorexia ($P < 0.0031$)
193 and to receive specific treatment ($P < 0.014$) than the others.

194

195 **DISCUSSION**

196 To the authors' knowledge, this is the first study on cryptosporidiosis symptomatology in hospitalized
197 neonatal foals caused by *C. parvum* and *Cryptosporidium* horse genotype.

198 There are few clinical studies about cryptosporidiosis in hospitalized foals; two of them describe
199 cryptosporidial infection in immunodeficient Arabian foals [4,11] and both demonstrated that foals
200 with Severe Combined Immunodeficiency (SCID) developed severe diarrhea and persistent
201 cryptosporidial infection following experimental challenge with *C. parvum* oocysts [11].

202 In the prospective study, the prevalence of *Cryptosporidium* spp. in hospitalized foals was 11.2%, but
203 this finding cannot be compared with other epidemiological surveys performed on farms in healthy
204 foals and adult horses. In the authors' opinion, an equine intensive care unit could be considered a
205 high-risk setting due both to the presence of sick and immunocompromised foals and to the high
206 environmental resistance of the oocysts which are not inactivated by the most common disinfectants
207 and can persist for a long period of time.

208 There are only two case reports about cryptosporidial infection in foals: one in a 9 days old Arabian
209 colt infected by *C. parvum* bovine genotype [35] and another in an Arabian foal with SCID [36].

210 In the 2003, Grinberg et al. [5] reported a severe outbreak of foal diarrhea, caused by *C. parvum* cattle
211 genotype, in a thoroughbred farm in New Zealand that lasted for one month and, during that period,
212 nine foals suffered from acute, mild to severe disease, accompanied by dehydration and weakness.
213 Affected foals showed yellowish foul smelling diarrhea sometimes associated with fever et al., and
214 with FPT [35] or with SCID [4,11,36]. It probably happens because foals at birth are immunologically
215 naïve to environmental antigens and lack adaptive immunity; consequently, they are highly
216 susceptible to pathogens that rarely caused disease in adults [37,38].

217 In the present study, the presence of FPT was not related to the cryptosporidial infection as reported
218 by other authors [5,13,18]. Moreover, it seems that the cryptosporidial infection was not influenced
219 by the presence of concomitant diseases, since it was observed also in healthy foals.

220 In immunocompetent foals oocysts first appeared in faeces between 9 and 28 days after birth and the
221 mean onset of patency was 14 days after birth. The mean period of oocysts shedding was 7 days, the
222 mean age at which diarrhea was first observed was 10 days and the duration of diarrhea ranged from
223 1 to 8 days with means duration of 3 days [17]. In the present study, the mean period of oocyst
224 shedding was not considered because it was limited by the duration of hospitalization. The duration
225 of diarrhea was not significantly different between *C. parvum*, *Cryptosporidium* horse genotype and
226 cryptosporidial mixed infection, between diarrhoeic foals positive or negative for *Cryptosporidium*
227 spp. and it was similar to the one reported by Coleman et al. (1989) [17]. Only one foal affected by

228 PAS had *C. parvum* diarrhea lasting 23 days and, due to the poor clinical condition, it was euthanized,
229 while in a case reported by Imhasly et al. (2009) [35], the colt was discharged in good clinical
230 conditions after 10 days.

231 The most important findings emerging from the present study is the presence of decreased bowel
232 sounds before appearance of diarrhea and the anorexia. Physical evidence of diarrhea may not be seen
233 early in the course of the disease, whether it is mild or severe [2]. Two mechanisms are implicated in
234 the pathogenesis of intestinal ileus: inflammation and distention. Local inflammation is probably due
235 to the local overproduction of nitric oxide acid caused by the upregulation of inducible nitric oxide
236 synthase by resident macrophages. Nitric oxide is the inhibitory neurotransmitter of the
237 nonadrenergic, noncholinergic system [39,40]. Excessive distention results in inhibition of motility
238 within the distended segment of bowel [41,42]. In the authors' opinion, this phase of hypomotility
239 can mislead the clinician, who does not promptly recognize the incoming diarrhea and does not isolate
240 the foal. In this way, the infected foal may become a source of infection for the other hospitalized
241 foals and for the personnel. Also Xiao and Herd (1994) [43] suggested that the main source of
242 *Cryptosporidium* spp. infection in foal was infected foals, while mares were the major source of
243 *Giardia* infection. In fact, in a more recent study of Galuppi et al. (2015) [26], no mare was positive
244 to *Cryptosporidium* spp. This finding suggests the importance of confining hospitalized foals with
245 hypomotility and abdominal discomfort in isolation until the presumptive diagnosis.

246 Anorexia was significantly prevalent in foals affected by *C. parvum*. In fact, no one foal with
247 *Cryptosporidium* horse genotype and mixed infection stopped to nurse from the mare.

248 Previously, *Cryptosporidium* horse genotype was identified twice without clinical symptomatology,
249 one in a Prezewalski's wild horse foal in Prague Zoo [15], and in nine foals 1–10 weeks of age in
250 New York [16].

251 Restriction of oral intake of milk can be very useful in the treatment of neonatal or young foals with
252 diarrhea, since it can be exacerbated by the osmotic influences of milk intake. 'Resting' of the gastro-
253 intestinal tract to allow for recovery of damaged enterocytes can be a beneficial part of therapy.

254 Neonates must be deprived of milk intake cautiously, as glucose energy stores are limited at this age.
255 Parenteral nutrition may be necessary and it allows increased periods of time without milk intake [2].
256 In the present study, the necessity of treatment of the cryptosporidial diarrhea was significantly
257 prevalent in foals affected by *C. parvum*. Moreover all the 7 foals infected only by *Cryptosporidium*
258 horse genotype and the 4 foals with mixed infection were discharged, while, among *C. parvum*
259 infected foals, 3 were euthanized due to the poor clinical condition and another one died soon after
260 the admission. Also Grinberg et al. (2003) [5] reported that, during a severe outbreak of *C. parvum*
261 diarrhea, two foals died from the disease and a third was euthanized due to a severe condition despite
262 intensive treatments.

263 These results could suggest that *C. parvum* infection is more severe in foals, probably because they
264 may have developed host adaptations in response to the *Cryptosporidium* horse genotype or because
265 *C. parvum* is a more pathogenic strain.

266 In human and bovine, the species most affected, several specific treatments are suggested for
267 cryptosporidiosis, but their specificity and efficacy are not completely verified [44-45]. In this
268 population, all foals affected by *C. parvum* and the 50% of foals with mixed infection needed an
269 intensive supportive therapy, while this has been necessary only for the 28.6% of foals affected by
270 *Cryptosporidium* horse genotype.

271 In healthy foals, a frequent cause of diarrhea is attributed to the mare's post-foaling oestrus. "Foal
272 heat diarrhea" affects up to 80% of foals 5–15 days-old, which remain bright and alert, continue to
273 nurse, but produce soft to watery faeces; this diarrhea is usually self-limiting and rarely requires
274 treatment [46-48]. Viruses, bacteria or parasites are suspected of predisposing foals to "foal heat
275 diarrhea" or to represent a complication [6,46]. It might be supposed that *Cryptosporidium* horse
276 genotype could be the parasite that causes the foal heat diarrhea. However, cases of neonatal
277 cryptosporidiosis in foals might remain undiagnosed and managed as generic "foal heat diarrhea"
278 [6,13], supporting the widespread of the infection.

279 In conclusion, the appropriate identification of potentially infected animal provides the basis to
280 prevent the widespread of infection to other foals, especially if hospitalized in intensive therapy.
281 Since healthy and asymptomatic foals can shed oocysts of *Cryptosporidium* horse genotype, students
282 and staff should always wear the personal protective equipment to avoid zoonotic infection.

283 **Acknowledgements**

284 This study was funded by "Progetti competitivi per Ricercatori" grant of Department of Veterinary
285 Medical Sciences (DIMEVET), Alma Mater Studiorum - University of Bologna, Budget integrato
286 2011.

287

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