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ABSTRACT

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

Keywords: foal, Cryptosporidium, diarrhea, zoonosis.

INTRODUCTION

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Experimental design

The present study was divided into 2 parts:

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

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

Data collection

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

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

Statistical analysis

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

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

RESULTS

Retrospective study

In the 2007 foaling season, the onset of clinical signs of cryptosporidiosis in the 6 included foals 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 were born at EPU). None foal died or was euthanized and the median hospitalization length was 35 ±5.1 days (range 17-45).

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

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

Prospective study

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

Eighteen/58 (1.1%) foals were born at EPU and 40/58 (68.9%) were referred. Seven foals were euthanized for poor clinical condition: 1/7 (14.3%) foal was positive to *C. parvum*, while none of the euthanized foals was positive for *Cryptosporidium* spp.

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

The onset of clinical signs in positive foals started at 5 ±0.9 days of life (range 1-13), after 2.5 ±0.7 days of hospitalization (range 0-6.5). Fifteen out of 58 animals (25.8%) tested positive for *Cryptosporidium* spp.: 4/15 (6.9%) for *C. parvum* (2 in 2012 and 2 in 2013), 7/15 (12%) for *Cryptosporidium* horse genotype (in 2012) and 4/15 (6.9%) 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 *Criptosporidium* horse genotype. Diarrhea, when present, showed similar
183 characteristics in both *Criptosporidium* 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].

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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