Molecular characterization of Cryptosporidium isolates from diarrheal dairy calves in France

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HAL Id: hal-03176056

https://hal.inrae.fr/hal-03176056

Submitted on 25 Oct 2021

1   **Molecular characterization of *Cryptosporidium* isolates from diarrheal dairy calves in**

2   **France**

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20 **Abstract**

21 *Cryptosporidium* is an obligate intracellular protist parasite infecting a wide range of

22 vertebrate hosts and causes significant intestinal disease in both animals and humans, as some

23 species are zoonotic. Cattle and especially calves have been identified as one of the most

24 common reservoirs of this protist. However, little is known about the genetics of

25 *Cryptosporidium* in calves in some regions of France. The aim of this study was to detect and

26 isolate *Cryptosporidium* spp. in faecal samples from naturally infected pre-weaned calves (≤

27 45 days-old) in France. A total of 35 diarrhoeic pre-weaned calf faecal samples were collected

28 from 26 dairy cattle farms in six departments (French administrative provinces).

29 *Cryptosporidium* presence was established by microscopically screening samples for oocytes

30 with an immunofluorescent (DFA) staining method. DFA-positive samples were then

31 analysed by PCR-RFLP and *18S rRNA* gene sequencing to determine species.

32 *Cryptosporidium parvum*-positive samples were subtyped via nested PCR analysis of a partial

33 fragment of the 60 kDa glycoprotein (*gp60*) gene product. Data were then integrated into

34 phylogenetic tree analysis. DFA revealed the presence of *Cryptosporidium* oocysts in 31 out

35 of 35 (88%) samples. Combined with *18S rRNA* gene analysis results, *C. parvum* was

36 detected in 30 samples. Subtyping analysis in 27/30 samples (90%) of the *C. parvum* isolates

37 revealed two zoonotic subtype families, IIa (24/27) and IId (3/27). Four subtypes were

38 recognised within the subtype family IIa, including the hypertransmissible IIaA15G2R1

39 subtype that is the most frequently reported worldwide (21/27), IIaA17G3R1 (1/27),

40 IIaA17G1R1 (1/27), and IIaA19G1R1 (1/27). Two subtypes were recognised within the IId

41 subtype family including IIdA22G1 (2/27) and IIdA27G1 (1/27). These findings illustrate the

42 high occurrence of *Cryptosporidium* in calves in dairy herds and increase the diversity of

43 molecularly characterised *C. parvum* isolates with the first description of IIaA17G3R1,

44 IIaA19G1R1, and IId subtypes in France. The presence of zoonotic *C. parvum* subtype

families (IIa, IId) in this study suggests that pre-weaned calves are likely to be a significant reservoir of zoonotic *C. parvum*, and highlights the importance of animal to human cryptosporidiosis transmission risk. Further molecular studies in calves and small ruminants from other French regions are required to better understand the epidemiology of cryptosporidiosis in France.

67   **1. Introduction**

68   *Cryptosporidium* is an obligate intracellular protist parasite infecting a wide range of

69   vertebrate hosts, including humans (Bouzid et al., 2013). The clinical importance of

70   *Cryptosporidium* spp. was highlighted when this parasite was associated with both waterborne

71   and foodborne outbreaks (Efstratiou et al., 2017; Ryan et al., 2018). Currently, more than 30

72   species have been described and validated (Osman et al., 2017). However, due to strong inter-

73   species similarities in microscopic size and shape, species within this genus require additional

74   identification via genetic characterisation. Molecular biology techniques have enabled the

75   description of species that are highly host-specific, as well as others that are capable of

76   infecting many hosts. *Cryptosporidium parvum* is considered to be the most prevalent species

77   worldwide and a major zoonotic transmission risk (Xiao, 2010). Using molecular approaches

78   to genetically characterise *Cryptosporidium* spp. has facilitated an improved understanding of

79   cryptosporidiosis epidemiology (Xiao, 2010). Subtype analysis using the *C. parvum* 60 kDa

80   glycoprotein locus (*gp60*) has revealed both human- and zoonotic-specific subtypes

81   (Sulaiman et al., 2005).

82   Within this context, cattle—particularly pre-weaned dairy calves—are recognised as common

83   hosts of *Cryptosporidium* spp., with both high infection rates and extremely high rates of

84   oocyst excretion in the faeces (Thomson et al., 2017). Cryptosporidiosis in neonatal calves is

85   mainly due to *C. parvum* infection, and is characterised by diarrhoea, dehydration, delayed

86   growth, and weight loss, resulting in considerable economic losses associated with calf

87   morbidity and mortality (Olson et al., 2004; Thomson et al., 2017). In addition, young calves

88   have been considered as a potential source of human cryptosporidiosis in several outbreaks

89   (Xiao, 2010). Besides *C. parvum*, cattle can also be infected by bovine specific species

including *C. bovis*, *C. andersoni* and *C. ryanae* with a marked aged-related pattern (Fayer et al., 2006; Santín et al., 2008). Asymptomatic adult cattle can also be sporadically infected by other species including *C. felis* (Cardona et al., 2015). It is well known that not all *Cryptosporidium* species excreted by cattle are zoonotic. Consequently, the molecular characterization of these species is vital to better understanding cattle cryptosporidiosis epidemiology and the zoonotic potential of specific *Cryptosporidium* isolates (Fayer et al., 2000).

Since the first report of the prevalence of bovine cryptosporidiosis in suckling (50-95%) and dairy calves (16.8-51,8%) in France in 1999 (Naciri et al., 1999), many cryptosporidiosis epidemiology studies have been solely based on faecal sample microscopy reporting different prevalences: 41.5 % (Delafosse et al., 2015) and 17.9 % (Lefay et al., 2000). However, these traditional staining or flotation screening methods can lead to suboptimal *Cryptosporidium* detection especially with reduced oocyst shedding intensity thus underestimating the real parasitic prevalence in herds. Thus far, only a few studies have used molecular tools to characterise *Cryptosporidium* spp. in cattle in 15 of the 95 metropolitan French departments (administrative regions): Allier, Côtes-d'Armor, Ille-et-Vilaine, Landes, Mayenne, Morbihan, Moselle, Orne, Pas-de-Calais, Puy-de-Dôme, Pyrénées-Atlantiques, Hautes-Pyrénées, Deux-Sèvres, Tarn, and Vendée (Follet et al., 2011; Ngouanesavanh et al., 2006; Rieux et al., 2014, 2013b, 2013c, 2013a). Little is known about which *Cryptosporidium* spp. infects calves in other French departments. However, subtype analysis solely on the basis of *C. parvum gp60* amplicons is insufficient (Follet et al., 2011; Rieux et al., 2014, 2013c, 2013a), thus there is a strong need for more molecular epidemiological data on French bovine cryptosporidiosis to fully determine the breadth of *C. parvum* genetic diversity. Thus, the aim of this study was to characterise *Cryptosporidium* isolates in calves from different French departments: Allier, Ardèche, Côte-d'Or, Moselle, Saône-et-Loire, and Yonne. Furthermore, through genetic

115 characterization, this study led the authors to investigate the potential of calves as a zoonotic

116 reservoir for human infection.

117 **Materials and methods**

118 **1.1. Sample collection and study design**

119 In order to generate a global understanding of the different *Cryptosporidium* species and *C.*

120 *parvum* genotypes in calf populations, mostly from uninvestigated French departments, a total

121 of 35 unique faecal samples were collected from pre-weaned calves ($\leq$ 45 days-old) on 26

122 dairy farms (with or without high density breeding, and/or the presence of diarrhoea), located

123 in six departments, between November 2017 and April 2018, with one sample per studied

124 animal. In order to perform anonymous sampling, farms were arbitrarily numbered from E1 to

125 E26 and collected faecal samples were labelled B1 to B35. A map (**Figure 1**) representing the

126 origin of the samples was edited using *Cartes et Données-* ® Articque

127 (https://www.articque.com/solutions/cartes-et-donnees/). Samples were directly collected

128 from the rectum using plastic gloves and transferred into a sterile plastic container. Samples

129 were maintained at 4°C for a maximum of 48 h prior to processing and analysis. For each

130 animal, the sampling date, animal identification number, age, and faecal score were recorded.

131 Faecal consistency was scored on a scale from 0 to 4 (0: normal without mucus; 1: pasty and

132 thick, formed or not, viscous 2: creamy; 3: semi-fluid; 4: liquid) as modified from Koudela

133 and Jirí (1997). The apparent occurrence (percentage of *Cryptosporidium* infection) was

134 calculated by dividing the number of *Cryptosporidium*-positive calves as assessed with

135 different techniques by the total number of tested animals, multiplied by 100.

136 **1.2. Microscopy**

137 One gram of faeces was either suspended in 10 mL of distilled water or concentrated using

138 the diethyl ether-PBS concentration method as previously described (Castro-Hermida et al.,

139 2005). The supernatant was removed and the pellet was resuspended in distilled water up to

140 500 µL. *Cryptosporidium* oocysts were detected from 20 µL of both concentrated and non-

141 concentrated oocyst solutions via direct immunofluorescence assay (DFA) using the

142 commercial Merifluor *Cryptosporidium/Giardia* immunofluorescence assay (Meridian

143 Diagnostics, Inc., Milano, Italy) as indicated by the manufacturer, and including previously

144 described modifications (Mammeri et al., 2018). Stained slides were observed with a Leica

145 fluorescent microscope using the Leica Application Suite software (version 4.5.0; Leica

146 Microsystems; Inc., Switzerland) at x40 magnification. The number of *Cryptosporidium*

147 oocysts per gram of faeces (OPG) was obtained by multiplying the total number of oocysts by

148 the dilution factor.

149 **1.3. DNA extraction**

150 Genomic DNA was extracted from 200 µL of concentrated oocysts using the QIAmp DNA

151 Stool Mini Kit (Qiagen, France) according to manufacturer's instructions. Briefly, samples

152 were suspended in lysis buffer and the oocysts were disrupted by subjecting them to an

153 additional step of 3 freeze-thaw cycles (freezing in liquid nitrogen for 1 min followed by

154 heating in a 90˚C water bath for 1 min) before DNA extraction. DNA samples were then

155 stored at -20°C until molecular analysis.

156 **1.4. *Cryptosporidium* genotyping with nested *18S rRNA* and *gp60* PCR**

157 *Cryptosporidium* species were further analysed in DFA-positive samples with nested PCR

158 which amplified a 847 bp fragment from the *18S rRNA* gene (Xiao et al., 1999).

159 Amplification products were subsequently visualised by electrophoresis in 2% agarose gels

160   stained with ethidium bromide (0.5 µg/mL). *Cryptosporidium* species were identified by

161   performing restriction fragment length polymorphism (RFLP) analysis with *SspI* and *MboII*

162   endonucleases on *18S rRNA* PCR products (New England BioLabs, France) as previously

163   described (Feng et al., 2007). Fragments were subsequently visualised by electrophoresis in

164   3% MetaPhor Agarose (Ozyme, France) gels stained with ethidium bromide solution (0.5

165   µg/mL). For example, in the case of *C. parvum*, *SspI* and *MboII* would generate three bands

166   (449, 267, and 108 bp) and two bands (771 and 769 bp), respectively (Feng et al., 2007).

167   *C. parvum* samples were subtyped by nested PCR-sequence analysis of the partial *gp60* gene

168   sequence as previously described (Gatei et al., 2006). Amplification products were

169   subsequently visualised by electrophoresis in 2% agarose gels stained with ethidium bromide.

170   In each PCR reaction, both positive and negative control samples were included. The positive

171   control consisted of DNA extracted from 10^6 *C. parvum* Iowa strain oocysts (Waterborne Inc.,

172   New Orleans, Louisiana, USA) while the negative control was purified water. In addition,

173   Bovine Serum Albumin (BSA) was added to all PCR mixtures at the final concentration of

174   400 ng/µL as described before (Jiang et al., 2005).

175   **1.5.   DNA sequence analysis**

176   PCR products of the two target genes (*18S rRNA* and *gp60*) were sequenced on both strands

177   using internal primer sets by Eurofins Genomics (France) and Genoscreen (France),

178   respectively. Consensus sequences were edited using the BioEdit Sequence Alignment Editor

179   software and compared with published GenBank sequences using the freely-available Basic

180   Local Alignment Search Tool (BLAST) from the National Center for Biotechnology

181   Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Representative nucleotide

182   consensus sequences generated in this study were deposited into the GenBank database under

183   accession numbers as indicated in **Table 1**. *C. parvum* subtypes were recognised based on the

184   number of trinucleotide repeats (TCA or TCG) or rare (R) repeats in the *gp60* sequence as

185   previously described (Sulaiman et al., 2005).

186   **1.6. Statistical analysis**

187   The Pearson correlation test was used to analyse data by comparing infection occurrence with

188   animal age, faecal score, and *C. parvum* subtypes. The results were considered statistically

189   significant when the *P*-value was < 0.05.

190   **2. Results**

191   **2.1. DFA screening**

192   Among the 35 samples included in this study, microscopic examinations revealed the

193   presence of *Cryptosporidium* oocysts in 88.5% of the samples from pre-weaned calves

194   (31/35) (**Table 1**).

195   Immunofluorescent-positive samples indicated that calves excreted from 10^4 to 3.41×10^6 OPG

196   via direct oocyst detection, or from 1.25×10^2 to 2.23×10^6 OPG following oocyst sample

197   concentration (**Figure. 2**). Results indicated oocyst loss in 20 of 30 (66.66%) of the samples

198   (the small amount of faeces from the B26 sample was concentrated and used to perform DFA

199   and PCRs). No correlation existed between age and infection intensity when DFA was used to

200   directly detect oocysts in faeces ($P = 0.68$), whereas a significant correlation was observed

201   between the faecal consistency score and infection intensity using this method ($P = 0.03$).

202   **2.2. Molecular analysis of the *18S rRNA* gene**

203   The *18S rRNA* gene fragment was amplified from concentrated oocysts for all 31 DFA-

204   positive samples (100%). *C. parvum* was identified in 29 of 31 (93.54%) DNA samples by

205   sequence analyses of the *18S rRNA* PCR products, as sequencing results were not usable for

206   the two remaining samples (B11 and B16). *C. parvum* was the only species identified in 29 of

207 31 (93.54%) samples by *18S rRNA* PCR-RFLP, and no mixed infections were observed. PCR

208 amplification failed for two samples (B16 and B30). Combining *18 S rRNA* sequence analysis

209 and PCR-RFLP results indicated that *C. parvum* was detected in 30 of 31 samples (96.7%).

210 Both techniques returned negative results for the B16 sample.

211 **2.3. Molecular analysis of *gp60* gene**

212 Of the 30 *Cryptosporidium 18S rRNA* gene-positive samples, all isolates yielded a *gp60* PCR

213 product. Successful sequencing analysis of the *gp60* gene in 27 (90%) *Cryptosporidium*

214 isolates identified two subtype families: IIa and IId (**Table 1**). Twenty-four out of 27 isolates

215 (88.8%) belonged to subtype family IIa and the remaining three isolates (11.2%) belonged to

216 IId.

217 Four subtypes were recognised within the IIa subtype family including IIaA15G2R1 (21/27),

218 IIaA17G3R1 (1/27), IIaA17G1R1 (1/27), and IIaA19G1R1 (1/27); while two subtypes were

219 identified as part of the IId subtype family, including IIdA22G1 (2/27) and IIdA27G1 (1/27).

220 Three PCR product for sequencing of the *gp60* loci from isolate which was successfully

221 identified as *C. parvum* by PCR-RFLP of the *18S rRNA* (isolate B11), sequence analyses of

222 the *18S rRNA* PCR products (isolate B30), or both techniques (B20), were not usable.

223 Several samples could not be used for all analyses; isolate B11 with successful *C. parvum*

224 identification via *18S rRNA* PCR-RFLP had a failed *gp60* PCR; sequencing of *18S rRNA*

225 PCRs could not be performed on isolate B30; and neither technique could be performed on

226 the B20 isolate.

227 Among the five farms infected with *C. parvum* and which contained more than two calves in

228 this study, three farms carried a single subtype (E1, E2, E17), which in this case was

229 IIaA15G2R1. For the E4 herd (B10 and B11 samples), one of the samples was linked to the

230 subtype IIdA22G1, while the other sample could not be associated with a specific subtype as

231 it likely carried a mixed infection of at least two subtypes. In herd E23, two circulating

232 subtypes were identified from samples B31 and B32, corresponding to subtypes IIaA17G1R1

233 and IIaA15G2R1, respectively.

234 No correlation was reported between *C. parvum* subtype and age ($P = 0.33$), faecal score

235 ($P=0.4$), or infection intensity ($P=0.8$).

3. Discussion

237 Several different techniques currently exist to detect *Cryptosporidium* infection in animal

238 and/or human faecal samples (Chalmers and Katzer, 2013). In the present study, DFA

239 screening was used to detect the presence of *Cryptosporidium* oocysts. This technique is

240 recommended as a screening method in epidemiological studies (Thompson and Ash, 2016)

241 due to its high sensitivity and rapidity, however a major disadvantage is that it is unable to

242 differentiate between *Cryptosporidium* species (Amar et al., 2004). In our study, the apparent

243 occurrence was estimated at 88.6% using DFA as a diagnostic screening technique. This high

244 value suggests that *Cryptosporidium* is a common parasite in pre-weaned calves in France,

245 and is also similar to that seen in other French epidemiological studies. In the Brittany region

246 (north-west France), 70.4% (100/142) of studied calves were reported to be infected by

247 *Cryptosporidium* spp. (Follet et al., 2011) using *18S rRNA* nested-PCR techniques. In the

248 Deux-Sèvres department, a 92-100% occurrence of *Cryptosporidium* infection in calves was

249 reported using DFA (Rieux et al., 2014). High *Cryptosporidium* occurrence in calves has also

250 been reported in other countries (e.g. the USA and Vietnam) (Nguyen et al., 2007; Santín et

251 al., 2004). However, a relatively low occurrence was recently described in western France

252 where only 402 out of the 968 (41.5%) analysed samples were positive using the Ziehl-

253 Neelsen fuchsin staining method (Delafosse et al., 2015). A worldwide *Cryptosporidium*

254 occurrence ranging from 3.4 to 96.6% has been reported in calves as previously reviewed

255   (Thomson et al., 2017). This extraordinarily broad variation in *Cryptosporidium* infection

256   occurrence with extreme dissimilarities between countries may be due to geographical

257   distribution (location of study farms), climatic conditions, and different farm management

258   practices, but also may relate to study design (number of specimens collected, number of

259   studied farms, specimen collection season, the diagnostic method used, etc.). Some of these

260   factors may also influence *Cryptosporidium* transmission between animals (Bamaiyi and

261   Redhuan, 2016; Causapé et al., 2002; Maurya et al., 2013; Mohammed et al., 1999; Ranjbar

262   and Fattahi, 2017). In addition to substantial differences in farm management, the lower

263   average age of infected calves in the present study might account for the higher occurrence,

264   since *Cryptosporidium* tends to be more frequently present in younger calves aged less than

265   one month (Santín et al., 2004).

266   Our results also indicated that four samples out of 35 (11.4%) were DFA-negative for the

267   presence of *Cryptosporidium* oocysts despite reported neonatal diarrhoea. This could be due

268   to the presence of other diarrhoea-causing enteropathogens (infection by *Escherichia coli*,

269   Rotavirus, Coronavirus, or Coccidia) (Thomson et al., 2017).

270   DFA-positive faecal samples indicated that calves excreted from between 1×10^4 to 3.41×10^6

271   oocysts when DFA was performed directly on faeces. This high level of oocyst excretion

272   reflects that of other studies performed in France which report that oocyst excretion intensity

273   can reach 8×10^6 oocysts per gram of faeces in some calves (Rieux et al., 2013b). When we

274   compared our DFA method *with* oocyst concentration to that *without*, we observed oocyst loss

275   in 65.5% of samples, which has previously been reported for concentration steps. However,

276   these purification techniques are still acceptable for use in young ruminants because they

277   excrete such a large number of oocysts (Fayer et al., 2000). On the other hand, oocyst

278 concentration may facilitate PCR detection of *Cryptosporidium* by eliminating naturally-

279 occurring PCR inhibitors from faeces (Elwin et al., 2012).

280 We reported a strong correlation between the faecal consistency score and infection intensity,

281 as estimated by DFA performed directly on faeces. This result is in accordance with previous

282 studies (El-Khodery and Osman, 2008; Trotz-Williams et al., 2007) which have reported a

283 strong correlation between *Cryptosporidium* oocyst shedding and calf diarrhoea.

284 Nevertheless, it is important to compare infection intensity (oocyst shedding) with the species

285 or subtypes detected by PCR, and faecal consistency, when generating inferences about the

286 clinical impact of cryptosporidiosis. DFA results highlighted the absence of any correlation

287 between calf age, oocyst shedding intensity, and *C. parvum* subtypes. This observation could

288 be explained by the fact that animals were not all infected at the same time, at the same age,

289 and the infectious dose may not have been the same. In addition, when the intestinal

290 epithelium is severely damaged by *Cryptosporidium* infection, parasitic reproduction is

291 impaired, even though marked diarrhoeal clinical signs are observed.

292 Despite positive microscopic identification of oocysts in samples, PCR analysis of the two

293 *18S rRNA* and *gp60* target genes was only positive in 93.5% and 90% samples, respectively.

294 Although the nested PCR method has been described as very sensitive and specific (Bhat et

295 al., 2014), this failure to yield PCR products was due to the unsuccessful PCR amplification.

296 These false negative PCR results could be explained by many factors: low numbers of oocysts

297 in some samples, the presence of PCR inhibitors in faecal samples (haemoglobin, bilirubin,

298 and bile acids), failed extraction procedures, failed oocyst disruption and lysis, insufficient

299 DNA collected, or nucleic acid degradation (Johnson et al., 1995; Lantz et al., 1997;

300 McLauchlin et al., 1999; Yu et al., 2009). Thus far, four common *Cryptosporidium* species

301 have been identified in cattle: *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni,* but only *C.*

302   *parvum* is associated with clinical disease in neonatal calves, as older animals (> 6 weeks)

303   exhibit asymptomatic oocyst shedding (Silverlås et al., 2013). The recent observation of *C.*

304   *hominis* in symptomatic and asymptomatic calves in France (Razakandrainibe et al., 2018)

305   emphasises the importance of identifying which *Cryptosporidium* species is shed by calves.

306   The calves included in this study were all younger than 45 days, thus the finding that 100% of

307   animals were infected with *C. parvum* via *18S rRNA* gene based-PCR-RFLP was similar to

308   previous studies demonstrating high *C. parvum* occurrence in pre-weaned calves (Kvác et al.,

309   2006; Santín et al., 2004). Our data also confirms that neonatal cryptosporidial diarrhoea in

310   calves is primarily caused by *C. parvum* (de Graaf et al., 1999; Santín et al., 2004). In contrast

311   to a previous reports (Björkman et al., 2015), no mixed infections were detected in the present

312   study, which could be attributed to the significant *C. parvum* presence as opposed to other

313   *Cryptosporidium* species which may represent smaller subpopulations. Species-specific

314   multiplex PCR or real-time PCR could be used to detect any low-level infections (Tanriverdi

315   et al., 2003).

316   Current guidelines suggest that genetic characterization of *Cryptosporidium* isolates should be

317   based on two genetic loci and include at least one conserved *18S rRNA* gene, thus in our

318   study, the *18S rRNA* and *gp60* genes were targeted (Cacciò et al., 2005). Concordant results

319   were obtained for the majority of isolates, with only three exceptions where one or both PCRs

320   failed, possibly due to the above-cited reasons.

321   Sequence analysis of the *gp60* gene showed that four *C. parvum* IIa subgenotype groups exist;

322   IIaA15G2R1, IIaA17G3R1, IIaA17G1R1, and IIaA19G1R1. These results concur with other

323   studies demonstrating that *C. parvum* IIa is a common subtype family in humans as well as

324   calves. Thus, this subtype family is considered to be potentially zoonotic and transmissible

325   from livestock (Xiao, 2010).

326   The predominant IIaA15G2R1 subtype has previously been reported as the most prevalent in

calves and humans in many countries (Aita et al., 2015; Alves et al., 2006; Danišová et al., 2016; Díaz et al., 2013; Mawly et al., 2015; Soba and Logar, 2008; Wielinga et al., 2008; Xiao, 2010), including France (Follet et al., 2011; Rieux et al., 2014, 2013c, 2013a), thus highlighting the zoonotic potential of calf reservoirs. It seems that the IIaA15G2R1 *C. parvum* subtype is hypertransmissible, which may explain its predominance (Feng et al., 2018). Future studies are needed to determine whether this subtype demonstrates greater infectivity and to what degree, or whether subtype predominance is due to a restricted available host.

Genotyping enabled the identification of relatively less dominant subtypes (IIaA17G1R1 in the Allier department, IIaA17G3R1 in the Moselle, and IIaA19G1R1 in the Ardèche). The IIaA17G1R1 subtype has previously been described in French beef cattle calves (Follet et al., 2011), as well as in many other countries such as Argentina (Tomazic et al., 2013), Estonia (Santoro et al., 2018), and the USA (Xiao et al., 2007), for example. Our study is the first to report the presence of IIaA17G3R1 and IIaA19G1R1 subtypes in French calves.

Only two *C. parvum* subtypes belonged to the IId family group (IIdA22G1 and IIdA27G1). In contrast to results from China (Cai et al., 2017; Feng and Xiao, 2017), the occurrence of this zoonotic IId family group in calves is rare in Europe and seems likely to have spread from Western Asia to other regions including France (Wang et al., 2014). This subtype family was described in European countries, such as in calves from Italy (Díaz et al., 2018), Belgium (Geurden et al., 2007), but never previously reported in France. Thus, the present study is the first to report the presence of the IId subtype in French calves. It appears that this subtype family is not restricted to a few farms, but could be easily transmitted to other animals (predominantly young ruminants) or to humans (Wang et al., 2014; Xiao, 2010).

In our study, only two farms were infected with more than one *C. parvum* family subtype. Mixed *C. parvum* subtype infections have previously been reported in studies with high

351 sampling numbers. In fact, it is possible that a range of *C. parvum* subtypes were circulating

352 in the same farm, but remained undetected when only one sampling was performed from each

353 animal (Mawly et al., 2015; Xiao et al., 2007). Similar studies on a larger geographic scale,

354 with greater numbers and horizontal sampling, are necessary to increase our understanding of

355 cryptosporidiosis transmission dynamics in calves.

356 **4.   Conclusion**

357 In conclusion, our findings demonstrate that young calves are a potential reservoir for

358 different *C. parvum* subtypes. The high occurrence of zoonotic *C. parvum* subtype family

359 infections (IIa, IId) in pre-weaned French calves confirms that calves should be considered as

360 a real source of infection and a potential zoonotic reservoir for human infections. Our results

361 also demonstrate that the *Cryptosporidium* population detected in France is more diverse than

362 previous studies would suggest. As a consequence, molecular studies in other regions,

363 including in calves and small ruminants, are needed to improve our understanding of

364 cryptosporidiosis epidemiology and *C. parvum* subtype diversity in France.

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633 **Figures**

634 **Figure 1. Geographical map of pre-weaned calf faecal sampling locations in French**

635 **departments.** The number of samples collected from each department (administrative

636 department number-department name) was: 03-Allier: n=18, 07-Ardèche: n= 2, 21-Côte-d'Or:

637 n=6, 57-Moselle: n=1, 71-Saône-et-Loire: n=1, and 89-Yonne: n=7.
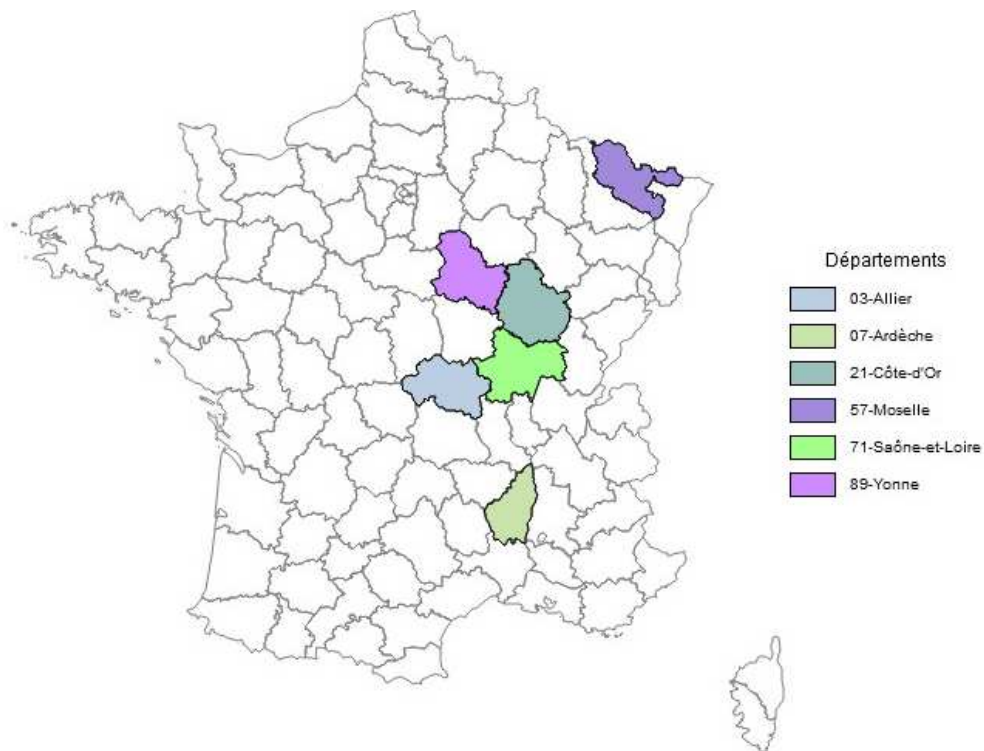
638

Figure 2. Pre-weaned calf oocyst excretion. Samples are classified from the youngest calves (4 days-old) to the oldest calf (45 days-old). DFA: Immunofluorescence Assay. OC: oocyst concentration. OPG: oocysts per gram of faeces.
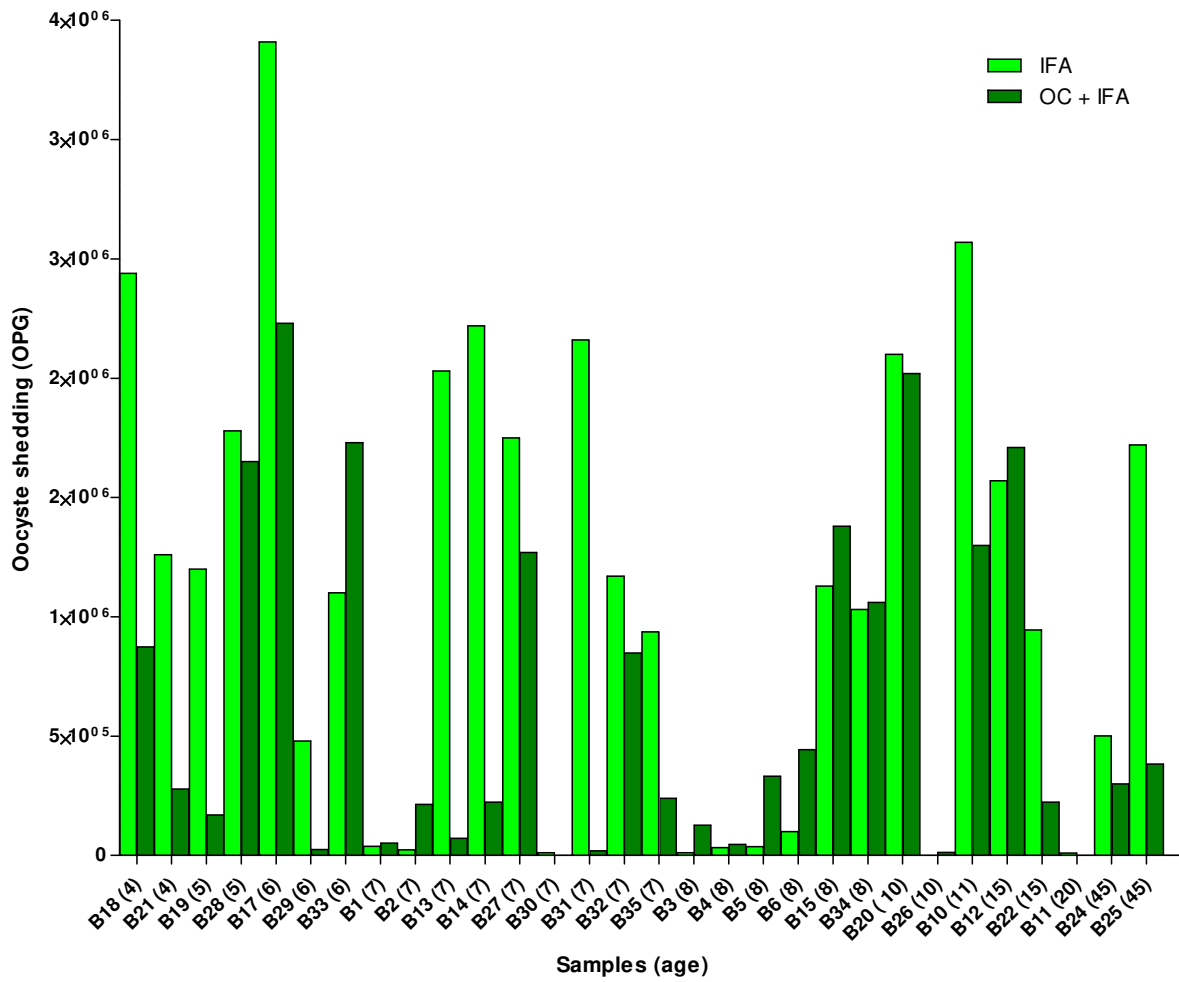
Table 1. Molecular characterization of *Cryptosporidium* from clinically-affected pre-weaned French calves, including age and faecal consistency data. n=total number of samples; 18S=18S ribosomal RNA; gp60=60kDa glycoprotein; NA=No Amplification; NU= Non-Usable; ND=not done. Department number: 03: Allier, 07: Ardèche, 21: Côte-d'Or, 57: Moselle, 71: Saône-et-Loire, 89: Yonne.

Samples (n=35)	Department	Herd	Age (days)	Faecal score	Sequencing (*18S rRNA*)	Access number (*18S rRNA*)	PCR (*18S rRNA*) + RFLP	Sequencing (*gp60*)	Access number (*gp60*)
B1	21	E1	7	3	*C. parvum*	MK014763	*C. parvum*	IIaA15G2R1	MK109829
B2	21		7	4	*C. parvum*	MK014764	*C. parvum*	IIaA15G2R1	MK109830
B3	21	E2	8	4	*C. parvum*	MK014765	*C. parvum*	IIaA15G2R1	MK109831
B4	21		8	2	*C. parvum*	MK014766	*C. parvum*	IIaA15G2R1	MK109832
B5	21		8	1	*C. parvum*	MK014767	*C. parvum*	IIaA15G2R1	MK109833
B6	21		8	2	*C. parvum*	MK014768	*C. parvum*	IIaA15G2R1	MK109834
B7	89	E3	30	4	**ND**	/	**ND**	**ND**	/
B8	89		30	3	**ND**	/	**ND**	**ND**	/
B9	89		30	1	**ND**	/	**ND**	**ND**	/
B10	89	E4	11	4	*C. parvum*	MK014769	*C. parvum*	IIdA22G1	MK109835
B11	89		20	2	**NU**	/	*C. parvum*	**NU**	/
B12	07	E5	15	4	*C. parvum*	MK014770	*C. parvum*	IIaA15G2R1	MK109836
B13	03	E6	7	4	*C. parvum*	MK014771	*C. parvum*	IIaA15G2R1	MK109837
B14	07	E7	7	4	*C. parvum*	MK014772	*C. parvum*	IIaA19G1R1	MK109838
B15	03	E8	8	4	*C. parvum*	MK014773	*C. parvum*	IIaA15G2R1	MK109839
B16	03	E9	13	NR	**NU**	/	**NA**	**NU**	/
B17	03	E10	6	4	*C. parvum*	MK014774	*C. parvum*	IIaA15G2R1	MK109840
B18	71	E11	4	4	*C. parvum*	MK014775	*C. parvum*	IIdA22G1	MK109841
B19	57	E12	5	3	*C. parvum*	MK014776	*C. parvum*	IIaA17G3R1	MK109842
B20	03	E13	10	2	*C. parvum*	MK014777	*C. parvum*	**NU**	/
B21	03	E14	4	4	*C. parvum*	MK014778	*C. parvum*	IIdA27G1	MK109843
B22	89	E15	15	4	*C. parvum*	MK014779	*C. parvum*	IIaA15G2R1	MK109844
B23	89	E16	15	4	**ND**	/	**ND**	**ND**	/
B24	03	E17	45	4	*C. parvum*	MK014780	*C. parvum*	IIaA15G2R1	MK109845
B25	03		45	4	*C. parvum*	MK014781	*C. parvum*	IIaA15G2R1	MK109846
B26	03	E18	10	NR	*C. parvum*	MK014782	*C. parvum*	IIaA15G2R1	MK109847
B27	03	E19	7	4	*C. parvum*	MK014783	*C. parvum*	IIaA15G2R1	MK109848
B28	03	E20	5	4	*C. parvum*	MK014784	*C. parvum*	IIaA15G2R1	MK109849
B29	03	E21	6	4	*C. parvum*	MK014785	*C. parvum*	IIaA15G2R1	MK109850
B30	03	E22	7	4	*C. parvum*	MK014786	**NA**	**NU**	/
B31	03	E23	7	4	*C. parvum*	MK014787	*C. parvum*	IIaA17G1R1	MK109851
B32	03		7	4	*C. parvum*	MK014788	*C. parvum*	IIaA15G2R1	MK109852
B33	03	E24	6	4	*C. parvum*	MK014789	*C. parvum*	IIaA15G2R1	MK109853

| B34 | 03 | E25 | 8 | 4 | *C. parvum* | MK014790 | *C. parvum* | IIaA15G2R1 | MK109854 |
| B35 | 03 | E26 | 7 | 4 | *C. parvum* | MK014791 | *C. parvum* | IIaA15G2R1 | MK109855 |