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Abstract

Cryptosporidium is an obligate intracellular protist parasite infecting a wide range of vertebrate hosts and causes significant intestinal disease in both animals and humans, as some species are zoonotic. Cattle and especially calves have been identified as one of the most common reservoirs of this protist. However, little is known about the genetics of *Cryptosporidium* in calves in some regions of France. The aim of this study was to detect and isolate *Cryptosporidium* spp. in faecal samples from naturally infected pre-weaned calves (≤ 45 days-old) in France. A total of 35 diarrhoeic pre-weaned calf faecal samples were collected from 26 dairy cattle farms in six departments (French administrative provinces). *Cryptosporidium* presence was established by microscopically screening samples for oocytes with an immunofluorescent (DFA) staining method. DFA-positive samples were then analysed by PCR-RFLP and *18S rRNA* gene sequencing to determine species. *Cryptosporidium parvum*-positive samples were subtyped via nested PCR analysis of a partial fragment of the 60 kDa glycoprotein (*gp60*) gene product. Data were then integrated into phylogenetic tree analysis. DFA revealed the presence of *Cryptosporidium* oocysts in 31 out of 35 (88%) samples. Combined with *18S rRNA* gene analysis results, *C. parvum* was detected in 30 samples. Subtyping analysis in 27/30 samples (90%) of the *C. parvum* isolates revealed two zoonotic subtype families, IIa (24/27) and IId (3/27). Four subtypes were recognised within the subtype family IIa, including the hypertransmissible IIaA15G2R1 subtype that is the most frequently reported worldwide (21/27), IIaA17G3R1 (1/27), IIaA17G1R1 (1/27), and IIaA19G1R1 (1/27). Two subtypes were recognised within the IId subtype family including IIdA22G1 (2/27) and IIdA27G1 (1/27). These findings illustrate the high occurrence of *Cryptosporidium* in calves in dairy herds and increase the diversity of molecularly characterised *C. parvum* isolates with the first description of IIaA17G3R1, 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.

Keywords: *Cryptosporidium parvum*, PCR-RFLP, *18S rRNA gene*, *gp60 subtype*, France, calves, zoonotic parasite.

66

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 (≤ 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**

One gram of faeces was either suspended in 10 mL of distilled water or concentrated using the diethyl ether-PBS concentration method as previously described (Castro-Hermida et al., 2005). The supernatant was removed and the pellet was resuspended in distilled water up to 500 µL. *Cryptosporidium* oocysts were detected from 20 µL of both concentrated and non-concentrated oocyst solutions via direct immunofluorescence assay (DFA) using the commercial Merifluor *Cryptosporidium/Giardia* immunofluorescence assay (Meridian Diagnostics, Inc., Milano, Italy) as indicated by the manufacturer, and including previously described modifications (Mammeri et al., 2018). Stained slides were observed with a Leica fluorescent microscope using the Leica Application Suite software (version 4.5.0; Leica Microsystems; Inc., Switzerland) at x40 magnification. The number of *Cryptosporidium* oocysts per gram of faeces (OPG) was obtained by multiplying the total number of oocysts by the dilution factor.

1.3. DNA extraction

Genomic DNA was extracted from 200 µL of concentrated oocysts using the QIAmp DNA Stool Mini Kit (Qiagen, France) according to manufacturer's instructions. Briefly, samples were suspended in lysis buffer and the oocysts were disrupted by subjecting them to an additional step of 3 freeze-thaw cycles (freezing in liquid nitrogen for 1 min followed by heating in a 90˚C water bath for 1 min) before DNA extraction. DNA samples were then stored at -20°C until molecular analysis.

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

Cryptosporidium species were further analysed in DFA-positive samples with nested PCR which amplified a 847 bp fragment from the *18S rRNA* gene (Xiao et al., 1999). 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

number of trinucleotide repeats (TCA or TCG) or rare (R) repeats in the *gp60* sequence as previously described (Sulaiman et al., 2005).

1.6. Statistical analysis

The Pearson correlation test was used to analyse data by comparing infection occurrence with animal age, faecal score, and *C. parvum* subtypes. The results were considered statistically significant when the *P*-value was < 0.05.

2. Results

2.1. DFA screening

Among the 35 samples included in this study, microscopic examinations revealed the presence of *Cryptosporidium* oocysts in 88.5% of the samples from pre-weaned calves (31/35) (**Table 1**).

Immunofluorescent-positive samples indicated that calves excreted from 10^4 to $3.41x10^6$ OPG via direct oocyst detection, or from $1.25x10^2$ to $2.23x10^6$ OPG following oocyst sample concentration (**Figure. 2**). Results indicated oocyst loss in 20 of 30 (66.66%) of the samples (the small amount of faeces from the B26 sample was concentrated and used to perform DFA and PCRs). No correlation existed between age and infection intensity when DFA was used to directly detect oocysts in faeces ($P = 0.68$), whereas a significant correlation was observed between the faecal consistency score and infection intensity using this method ($P = 0.03$).

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

The *18S rRNA* gene fragment was amplified from concentrated oocysts for all 31 DFA-positive samples (100%). *C. parvum* was identified in 29 of 31 (93.54%) DNA samples by sequence analyses of the *18S rRNA* PCR products, as sequencing results were not usable for the two remaining samples (B11 and B16). *C. parvum* was the only species identified in 29 of

31 (93.54%) samples by *18S rRNA* PCR-RFLP, and no mixed infections were observed. PCR amplification failed for two samples (B16 and B30). Combining *18 S rRNA* sequence analysis and PCR-RFLP results indicated that *C. parvum* was detected in 30 of 31 samples (96.7%). Both techniques returned negative results for the B16 sample.

2.3. Molecular analysis of *gp60* gene

Of the 30 *Cryptosporidium 18S rRNA* gene-positive samples, all isolates yielded a *gp60* PCR product. Successful sequencing analysis of the *gp60* gene in 27 (90%) *Cryptosporidium* isolates identified two subtype families: IIa and IId (**Table 1**). Twenty-four out of 27 isolates (88.8%) belonged to subtype family IIa and the remaining three isolates (11.2%) belonged to IId.

Four subtypes were recognised within the IIa subtype family including IIaA15G2R1 (21/27), IIaA17G3R1 (1/27), IIaA17G1R1 (1/27), and IIaA19G1R1 (1/27); while two subtypes were identified as part of the IId subtype family, including IIdA22G1 (2/27) and IIdA27G1 (1/27).

Three PCR product for sequencing of the *gp60* loci from isolate which was successfully identified as *C. parvum* by PCR-RFLP of the *18S rRNA* (isolate B11), sequence analyses of the *18S rRNA* PCR products (isolate B30), or both techniques (B20), were not usable.

Several samples could not be used for all analyses; isolate B11 with successful *C. parvum* identification via *18S rRNA* PCR-RFLP had a failed *gp60* PCR; sequencing of *18S rRNA* PCRs could not be performed on isolate B30; and neither technique could be performed on the B20 isolate.

Among the five farms infected with *C. parvum* and which contained more than two calves in this study, three farms carried a single subtype (E1, E2, E17), which in this case was IIaA15G2R1. For the E4 herd (B10 and B11 samples), one of the samples was linked to the 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).

236 **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 $1x10^4$ to $3.41x10^6$
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 $8x10^6$ 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

concentration may facilitate PCR detection of *Cryptosporidium* by eliminating naturally-occurring PCR inhibitors from faeces (Elwin et al., 2012).

We reported a strong correlation between the faecal consistency score and infection intensity, as estimated by DFA performed directly on faeces. This result is in accordance with previous studies (El-Khodery and Osman, 2008; Trotz-Williams et al., 2007) which have reported a strong correlation between *Cryptosporidium* oocyst shedding and calf diarrhoea. Nevertheless, it is important to compare infection intensity (oocyst shedding) with the species or subtypes detected by PCR, and faecal consistency, when generating inferences about the clinical impact of cryptosporidiosis. DFA results highlighted the absence of any correlation between calf age, oocyst shedding intensity, and *C. parvum* subtypes. This observation could be explained by the fact that animals were not all infected at the same time, at the same age, and the infectious dose may not have been the same. In addition, when the intestinal epithelium is severely damaged by *Cryptosporidium* infection, parasitic reproduction is impaired, even though marked diarrhoeal clinical signs are observed.

Despite positive microscopic identification of oocysts in samples, PCR analysis of the two *18S rRNA* and *gp60* target genes was only positive in 93.5% and 90% samples, respectively. Although the nested PCR method has been described as very sensitive and specific (Bhat et al., 2014), this failure to yield PCR products was due to the unsuccessful PCR amplification. These false negative PCR results could be explained by many factors: low numbers of oocysts in some samples, the presence of PCR inhibitors in faecal samples (haemoglobin, bilirubin, and bile acids), failed extraction procedures, failed oocyst disruption and lysis, insufficient DNA collected, or nucleic acid degradation (Johnson et al., 1995; Lantz et al., 1997; McLauchlin et al., 1999; Yu et al., 2009). Thus far, four common *Cryptosporidium* species 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

sampling numbers. In fact, it is possible that a range of *C. parvum* subtypes were circulating in the same farm, but remained undetected when only one sampling was performed from each animal (Mawly et al., 2015; Xiao et al., 2007). Similar studies on a larger geographic scale, with greater numbers and horizontal sampling, are necessary to increase our understanding of cryptosporidiosis transmission dynamics in calves.

4. Conclusion

In conclusion, our findings demonstrate that young calves are a potential reservoir for different *C. parvum* subtypes. The high occurrence of zoonotic *C. parvum* subtype family infections (IIa, IId) in pre-weaned French calves confirms that calves should be considered as a real source of infection and a potential zoonotic reservoir for human infections. Our results also demonstrate that the *Cryptosporidium* population detected in France is more diverse than previous studies would suggest. As a consequence, molecular studies in other regions, including in calves and small ruminants, are needed to improve our understanding of cryptosporidiosis epidemiology and *C. parvum* subtype diversity in France.

Conflicts of interest

The authors declare that they have no conflicting interests.

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Figures

Figure 1. Geographical map of pre-weaned calf faecal sampling locations in French departments. The number of samples collected from each department (administrative department number-department name) was: 03-Allier: n=18, 07-Ardèche: n= 2, 21-Côte-d'Or: n=6, 57-Moselle: n=1, 71-Saône-et-Loire: n=1, and 89-Yonne: n=7.

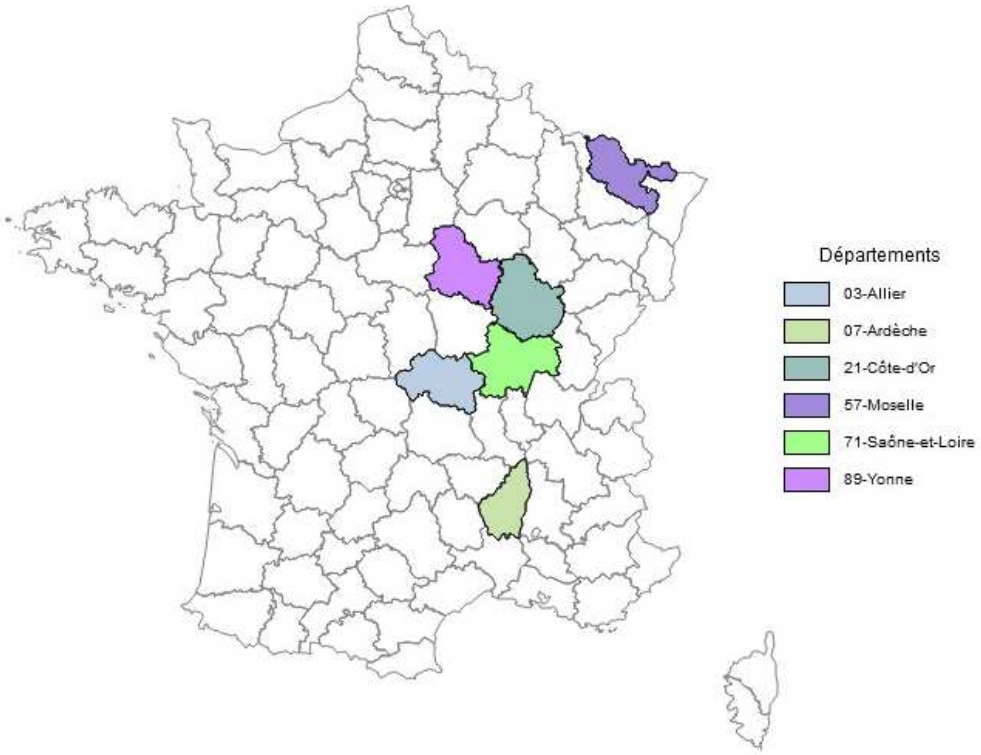

Figure 2. Pre-weaned calf oocyst excretion. Samples are classified from the youngest calves (4 days-old) to the oldest calf (45 days-old). DFA: Immunofluorescence Assay. OC: oocyst concentration. OPG: oocysts per gram of faeces.

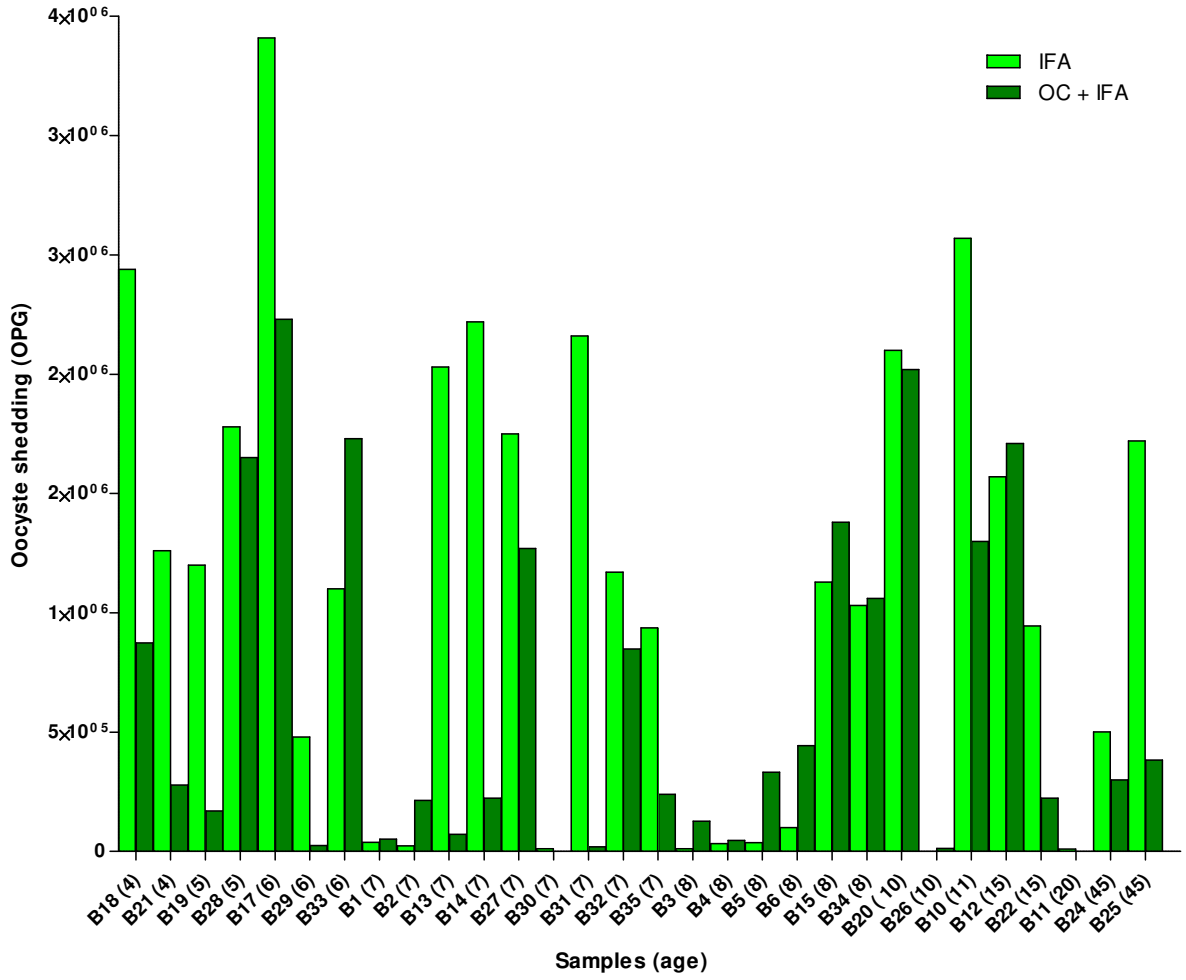

Table 1. Molecular characterization of *Cryptosporidium* from clinically-affected pre-weaned French calves, including age and faecal consistency data. n=total number of samples; 18S=18S ribosomal RNA; gp60=60kDa glycoprotein; NA=No Amplification; NU= Non-Usable; ND=not done. Department number: 03: Allier, 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