

Short Communication

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

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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 oocysts 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.

1. Introduction

Cryptosporidium is an obligate intracellular protist parasite infecting a wide range of vertebrate hosts, including humans (Bouزيد et al., 2013). The clinical importance of *Cryptosporidium* spp. was highlighted when this parasite was associated with both waterborne and foodborne outbreaks (Efstratiou et al., 2017; Ryan et al., 2018). Currently, > 30 species have been described and validated (Osman et al., 2017).

However, due to strong inter-species similarities in microscopic size and shape, species within this genus require additional identification via genetic characterization. Molecular biology techniques have enabled the description of species that are highly host-specific, as well as others that are capable of infecting many hosts. *Cryptosporidium parvum* is considered to be the most prevalent species worldwide and a major zoonotic transmission risk (Xiao, 2010). Using molecular approaches to genetically characterise *Cryptosporidium* spp. has facilitated an

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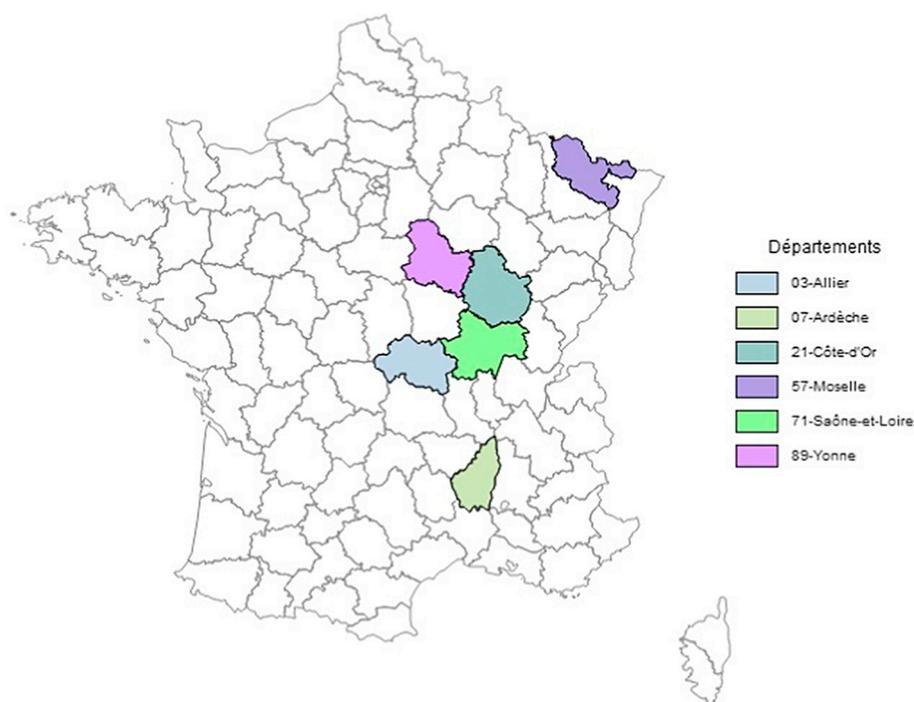


Fig. 1. Geographical map of pre-weaned calf faecal sampling locations in French departments. The number of samples collected from each department (administrative department number-department name) was: 03-Allier: n = 18, 07-Ardèche: n = 2, 21-Côte-d'Or: n = 6, 57-Moselle: n = 1, 71-Saône-et-Loire: n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

improved understanding of cryptosporidiosis epidemiology (Xiao, 2010). Subtype analysis using the *C. parvum* 60 kDa glycoprotein locus (*gp60*) has revealed both human- and zoonotic-specific subtypes (Sulaiman et al., 2005).

Within this context, cattle—particularly pre-weaned dairy calves—are recognised as common hosts of *Cryptosporidium* spp., with both high infection rates and extremely high rates of oocyst excretion in the faeces (Thomson et al., 2017). Cryptosporidiosis in neonatal calves is mainly due to *C. parvum* infection, and is characterised by diarrhoea, dehydration, delayed growth, and weight loss, resulting in considerable economic losses associated with calf morbidity and mortality (Olson et al., 2004; Thomson et al., 2017). In addition, young calves have been considered as a potential source of human cryptosporidiosis in several outbreaks (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 characterization, this study led the authors to investigate the potential of calves as a zoonotic reservoir for human infection.

2. Materials and methods

2.1. Sample collection and study design

In order to generate a global understanding of the different *Cryptosporidium* species and *C. parvum* genotypes in calf populations, mostly from uninvestigated French departments, a total of 35 unique faecal samples were collected from pre-weaned calves (≤ 45 days-old) on 26 dairy farms (with or without high density breeding, and/or the presence of diarrhoea), located in six departments, between November 2017 and April 2018, with one sample per studied animal. In order to perform anonymous sampling, farms were arbitrarily numbered from E1 to E26 and collected faecal samples were labelled B1 to B35. A map (Fig. 1) representing the origin of the samples was edited using *Cartes et Données* - Artique (<https://www.artique.com/solutions/cartes-et-donnees/>). Samples were directly collected from the rectum using plastic gloves and transferred into a sterile plastic container. Samples were maintained at 4 °C for a maximum of 48 h prior to processing and analysis. For each animal, the sampling date, animal identification number, age, and faecal score were recorded. Faecal consistency was scored on a scale from 0 to 4 (0: normal without mucus; 1: pasty and thick, formed or not, viscous; 2: creamy; 3: semi-fluid; 4: liquid) as modified from Koudela and Jirí (1997). The apparent occurrence (percentage of *Cryptosporidium* infection) was calculated by dividing the number of *Cryptosporidium*-positive calves as assessed with different techniques by the total number of tested animals, multiplied by 100.

2.2. 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 μ L. *Cryptosporidium* oocysts were detected from 20 μ L 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.

2.3. DNA extraction

Genomic DNA was extracted from 200 μ L 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.

2.4. *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 stained with ethidium bromide (0.5 μ g/mL). *Cryptosporidium* species were identified by performing restriction fragment length polymorphism (RFLP) analysis with *SspI* and *MboII* endonucleases on 18S rRNA PCR products (New England BioLabs, France) as previously described (Feng et al., 2007). Fragments were subsequently visualised by electrophoresis in 3% MetaPhor Agarose (Ozyme, France) gels stained with ethidium bromide solution (0.5 μ g/mL). For example, in the case of *C. parvum*, *SspI* and *MboII* would generate three bands (449, 267, and 108 bp) and two bands (771 and 769 bp), respectively (Feng et al., 2007).

C. parvum samples were subtyped by nested PCR-sequence analysis of the partial *gp60* gene sequence as previously described (Gatei et al., 2006). Amplification products were subsequently visualised by electrophoresis in 2% agarose gels stained with ethidium bromide.

In each PCR reaction, both positive and negative control samples were included. The positive control consisted of DNA extracted from 10⁶ *C. parvum* Iowa strain oocysts (Waterborne Inc., New Orleans, Louisiana, USA) while the negative control was purified water. In addition, Bovine Serum Albumin (BSA) was added to all PCR mixtures at the final concentration of 400 ng/ μ L as described before (Jiang et al., 2005).

2.5. DNA sequence analysis

PCR products of the two target genes (18S rRNA and *gp60*) were sequenced on both strands using internal primer sets by Eurofins Genomics (France) and Genoscreen (France), respectively. Consensus sequences were edited using the BioEdit Sequence Alignment Editor software and compared with published GenBank sequences using the freely-available Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Representative nucleotide consensus

sequences generated in this study were deposited into the GenBank database under 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).

2.6. 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.

3. Results

3.1. 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 10⁴ to 3.41 \times 10⁶ OPG via direct oocyst detection, or from 1.25 \times 10² to 2.23 \times 10⁶ OPG following oocyst sample concentration (Fig. 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* = .68), whereas a significant correlation was observed between the faecal consistency score and infection intensity using this method (*P* = .03).

3.2. 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 18S 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.

3.3. 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 IIDAA22G1 (2/27) and IIDAA27G1 (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

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 = 60 kDa 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	<i>C. parvum</i>	MK014763	<i>C. parvum</i>	IlaA15G2R1	MK109829
B2	21		7	4	<i>C. parvum</i>	MK014764	<i>C. parvum</i>	IlaA15G2R1	MK109830
B3	21	E2	8	4	<i>C. parvum</i>	MK014765	<i>C. parvum</i>	IlaA15G2R1	MK109831
B4	21		8	2	<i>C. parvum</i>	MK014766	<i>C. parvum</i>	IlaA15G2R1	MK109832
B5	21		8	1	<i>C. parvum</i>	MK014767	<i>C. parvum</i>	IlaA15G2R1	MK109833
B6	21		8	2	<i>C. parvum</i>	MK014768	<i>C. parvum</i>	IlaA15G2R1	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	<i>C. parvum</i>	MK014769	<i>C. parvum</i>	IIdA22G1	MK109835
B11	89		20	2	NU	/	<i>C. parvum</i>	NU	/
B12	07	E5	15	4	<i>C. parvum</i>	MK014770	<i>C. parvum</i>	IlaA15G2R1	MK109836
B13	03	E6	7	4	<i>C. parvum</i>	MK014771	<i>C. parvum</i>	IlaA15G2R1	MK109837
B14	07	E7	7	4	<i>C. parvum</i>	MK014772	<i>C. parvum</i>	IlaA19G1R1	MK109838
B15	03	E8	8	4	<i>C. parvum</i>	MK014773	<i>C. parvum</i>	IlaA15G2R1	MK109839
B16	03	E9	13	NR	NU	/	NA	NU	/
B17	03	E10	6	4	<i>C. parvum</i>	MK014774	<i>C. parvum</i>	IlaA15G2R1	MK109840
B18	71	E11	4	4	<i>C. parvum</i>	MK014775	<i>C. parvum</i>	IIdA22G1	MK109841
B19	57	E12	5	3	<i>C. parvum</i>	MK014776	<i>C. parvum</i>	IlaA17G3R1	MK109842
B20	03	E13	10	2	<i>C. parvum</i>	MK014777	<i>C. parvum</i>	NU	/
B21	03	E14	4	4	<i>C. parvum</i>	MK014778	<i>C. parvum</i>	IIdA27G1	MK109843
B22	89	E15	15	4	<i>C. parvum</i>	MK014779	<i>C. parvum</i>	IlaA15G2R1	MK109844
B23	89	E16	15	4	ND	/	ND	ND	/
B24	03	E17	45	4	<i>C. parvum</i>	MK014780	<i>C. parvum</i>	IlaA15G2R1	MK109845
B25	03		45	4	<i>C. parvum</i>	MK014781	<i>C. parvum</i>	IlaA15G2R1	MK109846
B26	03	E18	10	NR	<i>C. parvum</i>	MK014782	<i>C. parvum</i>	IlaA15G2R1	MK109847
B27	03	E19	7	4	<i>C. parvum</i>	MK014783	<i>C. parvum</i>	IlaA15G2R1	MK109848
B28	03	E20	5	4	<i>C. parvum</i>	MK014784	<i>C. parvum</i>	IlaA15G2R1	MK109849
B29	03	E21	6	4	<i>C. parvum</i>	MK014785	<i>C. parvum</i>	IlaA15G2R1	MK109850
B30	03	E22	7	4	<i>C. parvum</i>	MK014786	NA	NU	/
B31	03	E23	7	4	<i>C. parvum</i>	MK014787	<i>C. parvum</i>	IlaA17G1R1	MK109851
B32	03	E23	7	4	<i>C. parvum</i>	MK014788	<i>C. parvum</i>	IlaA15G2R1	MK109852
B33	03	E24	6	4	<i>C. parvum</i>	MK014789	<i>C. parvum</i>	IlaA15G2R1	MK109853
B34	03	E25	8	4	<i>C. parvum</i>	MK014790	<i>C. parvum</i>	IlaA15G2R1	MK109854
B35	03	E26	7	4	<i>C. parvum</i>	MK014791	<i>C. parvum</i>	IlaA15G2R1	MK109855

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 IlaA15G2R1. 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 it likely carried a mixed infection of at least two subtypes. In herd E23, two circulating subtypes were identified from samples B31 and B32, corresponding to subtypes IlaA17G1R1 and IlaA15G2R1, respectively.

No correlation was reported between *C. parvum* subtype and age ($P = .33$), faecal score ($P = .4$), or infection intensity ($P = .8$).

4. Discussion

Several different techniques currently exist to detect *Cryptosporidium* infection in animal and/or human faecal samples (Chalmers and Katzer, 2013). In the present study, DFA screening was used to detect the presence of *Cryptosporidium* oocysts. This technique is recommended as a screening method in epidemiological studies (Thompson and Ash, 2016) due to its high sensitivity and rapidity, however a major disadvantage is that it is unable to differentiate between *Cryptosporidium* species (Amar et al., 2004). In our study, the apparent occurrence was estimated at 88.6% using DFA as a diagnostic screening technique. This high value suggests that *Cryptosporidium* is a common parasite in pre-weaned calves in France, and is also similar to that seen in other French epidemiological studies. In the Brittany region (north-west France), 70.4% (100/142) of studied calves were reported

to be infected by *Cryptosporidium* spp. (Follet et al., 2011) using 18S rRNA nested-PCR techniques. In the Deux-Sèvres department, a 92–100% occurrence of *Cryptosporidium* infection in calves was reported using DFA (Rieux et al., 2014). High *Cryptosporidium* occurrence in calves has also been reported in other countries (e.g. the USA and Vietnam) (Nguyen et al., 2007; Santín et al., 2004). However, a relatively low occurrence was recently described in western France where only 402 out of the 968 (41.5%) analysed samples were positive using the Ziehl-Neelsen fuchsin staining method (Delafosse et al., 2015). A worldwide *Cryptosporidium* occurrence ranging from 3.4 to 96.6% has been reported in calves as previously reviewed (Thomson et al., 2017). This extraordinarily broad variation in *Cryptosporidium* infection occurrence with extreme dissimilarities between countries may be due to geographical distribution (location of study farms), climatic conditions, and different farm management practices, but also may relate to study design (number of specimens collected, number of studied farms, specimen collection season, the diagnostic method used, etc.). Some of these factors may also influence *Cryptosporidium* transmission between animals (Bamaiyi and Redhuan, 2016; Causapé et al., 2002; Maurya et al., 2013; Mohammed et al., 1999; Ranjbar and Fattahi, 2017). In addition to substantial differences in farm management, the lower average age of infected calves in the present study might account for the higher occurrence, since *Cryptosporidium* tends to be more frequently present in younger calves aged less than one month (Santín et al., 2004).

Our results also indicated that four samples out of 35 (11.4%) were DFA-negative for the presence of *Cryptosporidium* oocysts despite reported neonatal diarrhoea. This could be due to the presence of other

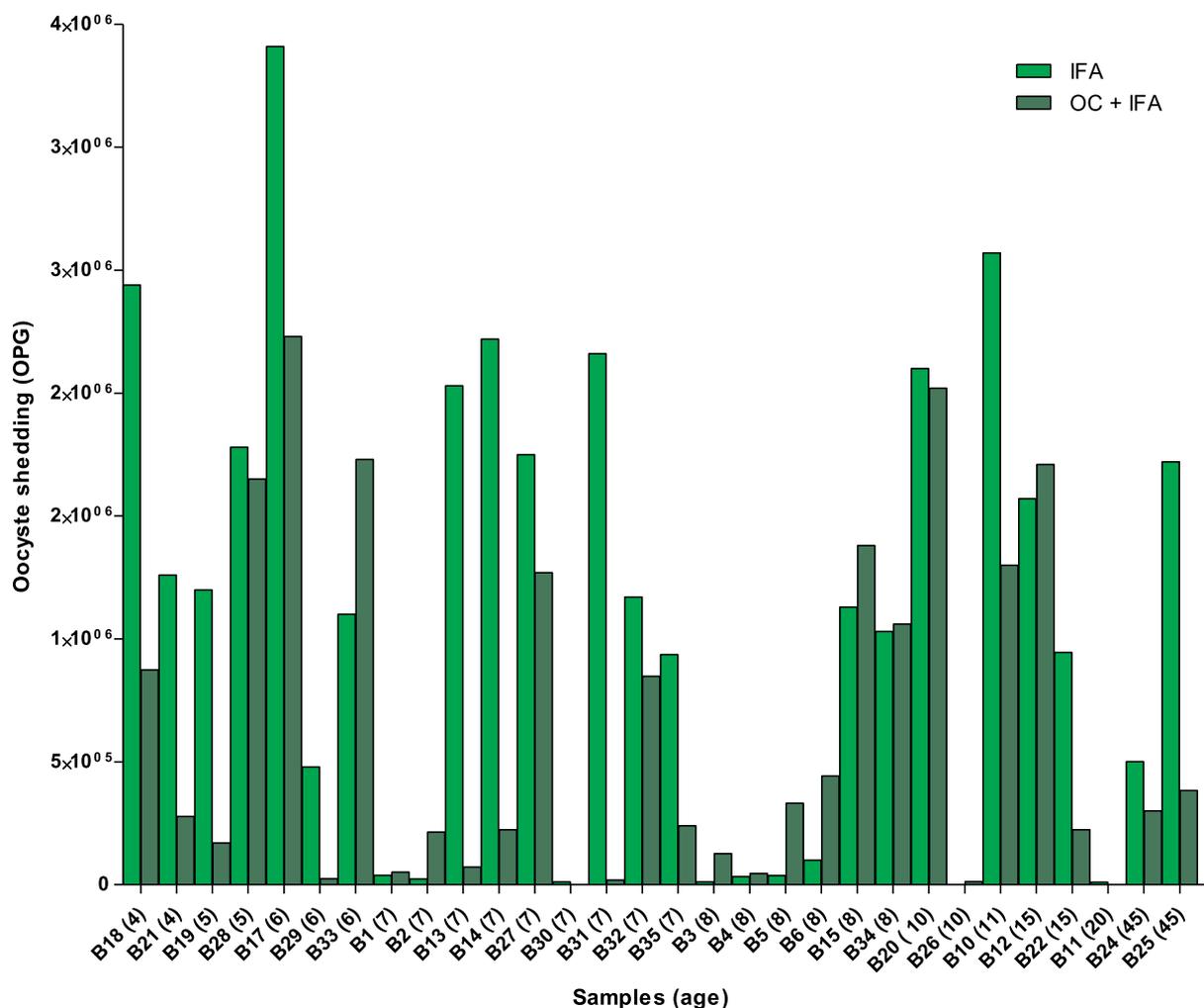


Fig. 2. Pre-weaned calf oocyst excretion. Samples are classified from the youngest calves (4 days-old) to the oldest calf (45 days-old). DFA: Immunofluorescence Assay. OC: oocyst concentration. OPG: oocysts per gram of faeces.

diarrhoea-causing enteropathogens (infection by *Escherichia coli*, Rotavirus, Coronavirus, or Coccidia) (Thomson et al., 2017).

DFA-positive faecal samples indicated that calves excreted from between 1×10^4 to 3.41×10^6 oocysts when DFA was performed directly on faeces. This high level of oocyst excretion reflects that of other studies performed in France which report that oocyst excretion intensity can reach 8×10^6 oocysts per gram of faeces in some calves (Rieux et al., 2013b). When we compared our DFA method with oocyst concentration to that without, we observed oocyst loss in 65.5% of samples, which has previously been reported for concentration steps. However, these purification techniques are still acceptable for use in young ruminants because they 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. parvum* is associated with clinical disease in neonatal calves, as older animals (> 6 weeks) exhibit asymptomatic oocyst shedding (Silverlås et al., 2013). The recent observation of *C. hominis* in symptomatic and asymptomatic calves in France (Razakandrainibe et al., 2018) emphasises the importance of identifying which *Cryptosporidium* species is shed by calves. The calves included in this study were all younger than 45 days, thus the finding that 100% of animals were infected with *C. parvum* via *18S rRNA* gene based-PCR-RFLP was similar

to previous studies demonstrating high *C. parvum* occurrence in pre-weaned calves (Kvác et al., 2006; Santín et al., 2004). Our data also confirms that neonatal cryptosporidial diarrhoea in calves is primarily caused by *C. parvum* (de Graaf et al., 1999; Santín et al., 2004). In contrast to a previous reports (Björkman et al., 2015), no mixed infections were detected in the present study, which could be attributed to the significant *C. parvum* presence as opposed to other *Cryptosporidium* species which may represent smaller subpopulations. Species-specific multiplex PCR or real-time PCR could be used to detect any low-level infections (Tanriverdi et al., 2003).

Current guidelines suggest that genetic characterization of *Cryptosporidium* isolates should be based on two genetic loci and include at least one conserved 18S rRNA gene, thus in our study, the 18S rRNA and *gp60* genes were targeted (Cacciò et al., 2005). Concordant results were obtained for the majority of isolates, with only three exceptions where one or both PCRs failed, possibly due to the above-cited reasons.

Sequence analysis of the *gp60* gene showed that four *C. parvum* IIA subgenotype groups exist; IIA15G2R1, IIA17G3R1, IIA17G1R1, and IIA19G1R1. These results concur with other studies demonstrating that *C. parvum* IIA is a common subtype family in humans as well as calves. Thus, this subtype family is considered to be potentially zoonotic and transmissible from livestock (Xiao, 2010).

The predominant IIA15G2R1 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 IIA15G2R1 *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 (IIA17G1R1 in the Allier department, IIA17G3R1 in the Moselle, and IIA19G1R1 in the Ardèche). The IIA17G1R1 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 IIA17G3R1 and IIA19G1R1 subtypes in French calves.

Only two *C. parvum* subtypes belonged to the IID family group (IID22G1 and IID27G1). 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.

5. 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.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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