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1 **First identification of *Cryptosporidium parvum* zoonotic subtype IIaA15G2R1 in**
2 **diarrheal lambs in France**

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16

17 **Abstract**

18 To date, no information is available about the presence of *Cryptosporidium* spp. in French
19 sheep, nor their potential role as zoonotic reservoirs. A total of 23 fecal samples were
20 collected from **diarrheic** lambs (< 11 days old) from seven randomly selected farms.
21 *Cryptosporidium*-oocysts were detected microscopically with Direct Immunofluorescence
22 Assays (DFA) in 23/23 (100%) of fecal samples. PCR-RFLP of the *18S rRNA* gene was used
23 to determine species in all samples, and only *Cryptosporidium parvum* was identified. Isolates
24 were subtyped by sequencing the 60 kDa glycoprotein (*gp60*) gene. Two zoonotic subtypes
25 within the IIa subtype family were identified, including IIaA15G2R1 (22/23) and
26 IIaA16G3R1 (1/23). This study reports for the first time the identification and genotyping of
27 zoonotic *C. parvum* subtypes from lambs in France. Sheep could thus play an important role
28 as potential reservoirs for this zoonotic protist.

29 **Key words:** *Cryptosporidium*; Lamb; Sheep; Zoonosis; France.

30

31 **Introduction**

32 *Cryptosporidium* is an obligate intracellular protist parasite infecting a wide range of
33 vertebrate hosts—including humans (Bouzid et al., 2013)—and poses a significant threat to
34 public health. Molecular approaches to genetically characterise *Cryptosporidium* spp. has
35 enhanced an improved understanding of cryptosporidiosis epidemiology (Xiao, 2010a).
36 Clinical symptoms of *Cryptosporidium* infection in young ruminants (calves, lambs, and goat
37 kids) include diarrhea, dehydration, delayed growth, and weight loss, often leading to death,
38 thus resulting in considerable economic losses associated with morbidity and mortality (de
39 Graaf et al., 1999). In addition, young ruminants have been considered as a potential source of
40 human cryptosporidiosis infection in several outbreaks (Xiao, 2010a).

41 Currently, more than 30 validated *Cryptosporidium* species have been described (Osman et
42 al., 2017). Besides *C. parvum*, six *Cryptosporidium* species have been identified in sheep
43 feces, including *C. ubiquitum*, *C. xiaoi*, *C. hominis*, *C. andersoni*, *C. fayeri*, and *C. suis*
44 (Paraud and Chartier, 2012). However, it is not yet known which specific *Cryptosporidium*
45 species/subtypes infect sheep in France. Thus far, many studies have characterized
46 *Cryptosporidium* at a molecular level in French calves (Follet et al., 2011; Ngouanesavanh et
47 al., 2006; Razakandrainibe et al., 2018; Rieux et al., 2014, 2013b, 2013c, 2013a) and goat
48 kids (Ngouanesavanh et al., 2006; Paraud et al., 2014; Rieux et al., 2013d). Little is known
49 about the presence of *Cryptosporidium* spp. in sheep, nor the role the animals may play as
50 reservoirs for these parasites. Therefore, the present work aimed to identify *Cryptosporidium*
51 at a molecular level in lambs from two different French departments (Tarn and Haute-
52 Vienne). Furthermore, through genetic characterization, this study led the authors to
53 investigate the potential of lambs as a zoonotic reservoir for human infection.

54

55 **Materials and Methods**

56 Between November 2018 to April 2019, 23 lamb rectal fecal samples were collected from 7
57 **randomly selected** farms across two French departments: Tarn and Haute-Vienne (Figure 1).
58 In order to perform anonymous sampling, farms were arbitrarily numbered from F1 to F7 and
59 collected stool samples were labelled O1 to O23. The farms included in this study all breed
60 mixed ruminants (cattle, sheep, and goats). Sampled lambs were less than 11 days old, and
61 presented with diarrhea. Fecal samples were individually collected from lambs in plastic
62 containers, and conserved at 4°C until analysis within one week.

63

64 **Microscopy screening**

65 All samples were concentrated from 1 g of original fecal matter as previously described
66 (Castro-Hermida et al., 2005), then screened for the presence of *Cryptosporidium* oocysts by
67 direct immunofluorescence assays (DFA) (MeriFluor® *Cryptosporidium/Giardia*, Meridian
68 Bioscience Europe, Milano, Italy) as indicated by the manufacturer, and including previously
69 described modifications (Mammeri et al., 2018).

70

71 **DNA extraction and PCR amplification**

72 Samples with positive DFA underwent genomic DNA extraction using the QIAamp DNA
73 Stool Mini Kit (Qiagen, France), according to manufacturer's instructions. To disrupt oocyst
74 walls, an initial step of ten freeze-thaw cycles was incorporated into the protocol as
75 previously described (Sahraoui et al., 2019).

76 To detect *Cryptosporidium* spp. in DFA-positive samples, nested PCR was used to amplify an
77 840 bp fragment of *18S rRNA* gene as previously described (Xiao et al., 1999).
78 *Cryptosporidium* species were identified by performing restriction fragment length

79 polymorphism (RFLP) analysis with *SspI* and *MboII* endonucleases on *18S rRNA* PCR
80 products (New England BioLabs, France) as previously described (Feng et al., 2007).
81 Comparison of band patterns with those described before (Feng et al., 2007) was used for the
82 identification of the different *Cryptosporidium* species.

83 *Cryptosporidium parvum* samples were subtyped by nested PCR-sequence analysis of the
84 partial 60 kDa glycoprotein locus (*gp60*), and all positive isolates were sequenced as
85 previously described (Alves et al., 2003). Briefly, *gp60*-PCR products were sequenced on
86 both strands using internal primer sets by Genoscreen (France). Consensus sequences were
87 edited using the BioEdit Sequence Alignment Editor software (version 5.0.6) and compared
88 with published GenBank sequences using the freely-available Basic Local Alignment Search
89 Tool (BLAST) from the National Center for Biotechnology Information (NCBI)
90 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). *C. parvum* subtypes were named using the
91 recommended nomenclature system (Sulaiman et al., 2005; Xiao, 2010b). All *gp60* sequences
92 generated in this study from O1 to O23 isolates were deposited into the Genbank database
93 with accession numbers MN037849-MN37871, respectively.

94 In each PCR reaction, both positive and negative control samples were included. The positive
95 control consisted of DNA extracted from 10^6 *C. parvum* Iowa strain oocysts (Waterborne Inc.,
96 New Orleans, Louisiana, USA) while the negative control was purified water.

97 **Results and Discussion**

98 *Cryptosporidium* species may pose a significant threat to public health. They are well-known
99 pathogens infecting both domesticated farm and companion animals. There is considerable
100 genetic diversity within *Cryptosporidium*, as 30 *Cryptosporidium* species with several
101 different subtypes have been described (Cacciò et al., 2005). However, little is known about
102 *Cryptosporidium* occurrence rates in small ruminants in France. For the first time, this study
103 describes the *C. parvum* *gp60* subtypes isolated from lamb feces samples in France.

104 In this study, DFA was used to screen for the presence of *Cryptosporidium* oocysts, prior to
105 genotyping with PCR. *Cryptosporidium* spp. were detected by DFA in 23/23 (100%) of fecal
106 samples from young diarrhoeic lambs (Table 1). Although the number of samples included in
107 this study is small, this study implicates the *Cryptosporidium* species as a neonatal diarrhea
108 agent, however, other intestinal pathogens (*Escherichia coli*, *Salmonella*, *Coccidia*...) that
109 were not investigated here, could also be diarrhea-causing agents in these lambs. DFA-
110 positive samples indicated that lambs excreted from between 2×10^3 to 9×10^7 oocysts per
111 gram of feces (OPG) via direct oocyst detection (Mean = 8×10^6). These results indicate a
112 high level of oocyst excretion, and are similar to a study performed in France which reported
113 oocyst excretion intensity reaching 8×10^6 oocysts per gram of feces in some calves (Rieux et
114 al., 2013b).

115 In this study, PCR-RFLP and sequence analysis of the *18S rRNA* gene confirmed that only the
116 *C. parvum* species was present in the lambs (23/23) (Table 1) (Figure 2). The success of the
117 PCR technique in all samples could be explained by the high excreted parasite load, which
118 may overcome the effects of any naturally-occurring PCR inhibitors in the feces. On the other
119 hand, oocyst concentration may also facilitate *Cryptosporidium* PCR detection by eliminating
120 those naturally-occurring PCR inhibitors (Elwin et al., 2012).

121 As already mentioned, *C. parvum* was the only species identified in this study, similar to
122 previous small ruminant studies in other countries (Drumo et al., 2012; Goma et al., 2007;
123 Maurya et al., 2013; Mueller-Doblies et al., 2008; Quilez et al., 2008; Tzanidakis et al., 2014).
124 However, even though the *C. xiaoi* species is often reported in small ruminants in other
125 countries, it was not identified in lambs in the current study (Paraud and Chartier, 2012).

126 Subtype analysis using the *C. parvum* 60 kDa glycoprotein locus (*gp60*) (Figure 2. C)
127 revealed both human- and zoonotic-specific subtypes (Sulaiman et al., 2005). In this study,

128 the dominant *C. parvum* isolate subtype present in the lambs was IIAA15G2R1 (n = 22/23),
129 while subtype IIAA16G3R1(1/23) was reported at lower rates (Table 1). Our results are
130 consistent with multiple other sheep studies. In fact, it has been reported that the *C. parvum*
131 IIA subtype family is dominant in countries such as the UK, Poland, and New Guinea
132 (Connelly et al., 2013; Kaupke et al., 2017; Koinari et al., 2014). The identified IIA subtypes
133 pose a real risk to public health, as this family is known to include many potentially zoonotic
134 subtypes (Xiao, 2010a). Of note, in other countries (Spain, Romania, and Australia) the IID
135 subtype family dominate (Díaz et al., 2015; Imre et al., 2013; Quilez et al., 2008; Yang et al.,
136 2014).

137 The predominant IIAA15G2R1 subtype has previously been reported as the most prevalent
138 subtype in calves and humans in many countries (Aita et al., 2015; Alves et al., 2006;
139 Danišová et al., 2016; Díaz et al., 2013; Mawly et al., 2015; Soba and Logar, 2008; Wielinga
140 et al., 2008; Xiao, 2010a), including France (Follet et al., 2011; Rieux et al., 2014, 2013c,
141 2013a), thus highlighting the zoonotic potential of lamb reservoirs. It seems that the
142 IIAA15G2R1 *C. parvum* subtype is hypertransmissible, which may explain its predominance
143 (Feng et al., 2018). Future studies are needed to determine whether this subtype is only
144 isolated from mixed-species breeding, or whether subtype predominance is due to one
145 restricted available host.

146 The IIAA16G3R1 *C. parvum* subtype has been identified in many studies of calves from
147 France (Follet et al., 2011; Razakandrainibe et al., 2018; Rieux et al., 2013a), and in
148 ruminants (calves, lambs, and goat kids) from other locations (Spain, Korea, Australia, and
149 Algeria) (Díaz et al., 2015; Lee et al., 2016; Nolan et al., 2009; Sahraoui et al., 2019). In
150 addition, human *Cryptosporidium* infections, including subtype IIAA16G3R1, have been
151 reported in patients from Denmark and Iran (Kiani et al., 2017; Stensvold et al., 2015).

152 Our results suggest that lambs may also be important reservoirs for *C. parvum* zoonotic
153 subtypes in France. Further investigations are required to determine whether this observation
154 holds true in other parts of the country on a larger geographic scale, preferably with larger
155 sample sizes from different French departments, and different farm management practices, to
156 better understand the epidemiology of cryptosporidiosis in lambs.

157 Sequencing of the *gp60* gene could demonstrate the presence of common subtype families in
158 humans as well as animals. This could provide more information about these potentially
159 zoonotic subtype families and their transmission from livestock.

160 **Conclusion**

161 In conclusion, our findings demonstrate that *C. parvum* infection is a common occurrence in
162 lambs. These data strongly suggest that lambs may be important reservoirs of zoonotic *C.*
163 *parvum* subtypes infecting humans in France. This is also the first report of *C. parvum*
164 subtype infections in French lambs, and could serve as baseline data for further investigations
165 to better understand cryptosporidiosis epidemiology and *C. parvum* subtype diversity in
166 France.

167 **Conflict of interest**

168 The authors declare that they have no conflicts of interest.

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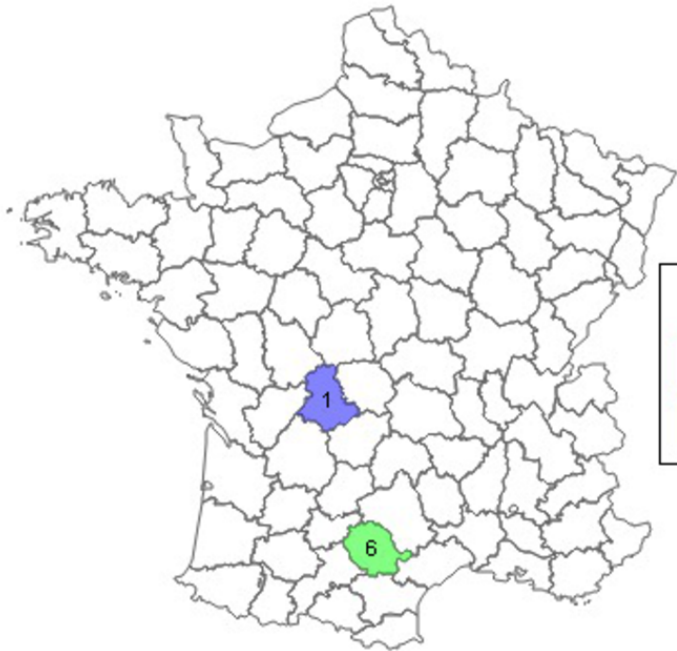
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1 **Figures**

2 **Figure 1.** Geographical map of pre-weaned lamb faecal sampling locations in French
3 departments.

4 The number of sampled farm from each French department (administrative department
5 number-department name) was: 81-Tarn: n=6, and 87-Haute-Vienne: n= 1. The map was
6 edited using *Cartes et Données*- ® Articque ([https://www.articque.com/solutions/cartes-et-](https://www.articque.com/solutions/cartes-et-donnees/)
7 [donnees/](https://www.articque.com/solutions/cartes-et-donnees/)).



French department



81-Tarn



87-Haute-Vienne



Table 1. Molecular characterization of *Cryptosporidium* from clinically-affected pre-weaned French lamb, including age and parasitic burden (oocysts shedding) data.

Sampled lambs and farms included in this study were arbitrary designed from O1 to O23, and from F1 to F7, respectively. Department number: 81: Tarn, and 87: Haute-Vienne; n = total number of samples from each farm; OPG: oocysts per gram of feces.

No. of samples (n = 23)	Department number	Farm (n)	Age (Days)	OPG	<i>C. parvum</i> subtype	Accession numbers	
O1	81 (Tarn)	F1 (1)	10	2 x 10 ³	IIaA15G2R1	MN37849	
O2		F2 (1)	10	8 x 10 ⁴		MN37850	
O12		F4 (1)	7	2 x 10 ⁷		MN37860	
O13			8	1 x 10 ⁶		MN37861	
O14			8	8 x 10 ⁶		MN37862	
O15		F5 (5)	8	3 x 10 ⁶		MN37863	
O16			8	9 x 10 ⁶		MN37864	
O17			8	7 x 10 ⁵		MN37865	
O18		F6 (1)	5	2 x 10 ⁶		IIaA15G2R1	MN37866
O19			5	9 x 10 ⁷		MN37867	
O20			5	3 x 10 ⁶		MN37868	
O21		F7 (5)	10	7 x 10 ⁶		IIaA15G2R1	MN37869
O22			8	3 x 10 ⁶		MN37870	
O23			6	1 x 10 ⁷		MN37871	
O3	87 (Haute-Vienne)		10	7 x 10 ⁵	IIaA15G2R1	MN37851	
O4			5	1 x 10 ⁶		MN37852	
O5			6	1 x 10 ⁶		MN37853	
O6			4	5 x 10 ⁵		MN37854	
O7		F3 (9)	6	1 x 10 ⁵		MN37855	
O8			2	1 x 10 ⁵		MN37856	
O9			2	2 x 10 ⁵		MN37857	
O10			5	5 x 10 ⁴		MN37858	
O11			6	8 x 10 ⁴		MN37859	