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▶ **To cite this version:**

Mohamed Mammeri, Lara Cartou, Aurélie Chevillot, Myriam Thomas, Christine Julien, et al.. First identification of Cryptosporidium parvum zoonotic subtype IIaA15G2R1 in diarrheal lambs in France. Veterinary Parasitology : Regional Studies and Reports, 2019, 18, 10.1016/j.vprsr.2019.100355 . hal-03176049

HAL Id: hal-03176049
https://hal.inrae.fr/hal-03176049v1

Submitted on 21 Jul 2022

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Version of Record: https://www.sciencedirect.com/science/article/pii/S2405939019302394
Manuscript_77ee81f7b17c97113cde93dcb5f4fb0e

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

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Abstract

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

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

Introduction

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

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

Materials and Methods

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

Microscopy screening

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

DNA extraction and PCR amplification

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

To detect *Cryptosporidium* spp. in DFA-positive samples, nested PCR was used to amplify an 840 bp fragment of *18S rRNA* gene as previously described (Xiao et al., 1999). *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). Comparison of band patterns with those described before (Feng et al., 2007) was used for the identification of the different *Cryptosporidium* species.

Cryptosporidium parvum samples were subtyped by nested PCR-sequence analysis of the partial 60 kDa glycoprotein locus (*gp60*), and all positive isolates were sequenced as previously described (Alves et al., 2003). Briefly, *gp60*-PCR products were sequenced on both strands using internal primer sets by Genoscreen (France). Consensus sequences were edited using the BioEdit Sequence Alignment Editor software (version 5.0.6) 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). *C. parvum* subtypes were named using the recommended nomenclature system (Sulaiman et al., 2005; Xiao, 2010b). All *gp60* sequences generated in this study from O1 to O23 isolates were deposited into the Genbank database with accession numbers MN037849-MN37871, respectively.

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

Results and Discussion

Cryptosporidium species may pose a significant threat to public health. They are well-known pathogens infecting both domesticated farm and companion animals. There is considerable genetic diversity within *Cryptosporidium*, as 30 *Cryptosporidium* species with several different subtypes have been described (Cacciò et al., 2005). However, little is known about *Cryptosporidium* occurrence rates in small ruminants in France. For the first time, this study 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 x 10^3 to 9 x 10^7 oocysts per
111 gram of feces (OPG) via direct oocyst detection (Mean = 8 x 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 x 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,

the dominant *C. parvum* isolate subtype present in the lambs was IIaA15G2R1 (n = 22/23), while subtype IIaA16G3R1(1/23) was reported at lower rates (Table 1). Our results are consistent with multiple other sheep studies. In fact, it has been reported that the *C. parvum* IIa subtype family is dominant in countries such as the UK, Poland, and New Guinea (Connelly et al., 2013; Kaupke et al., 2017; Koinari et al., 2014). The identified IIa subtypes pose a real risk to public health, as this family is known to include many potentially zoonotic subtypes (Xiao, 2010a). Of note, in other countries (Spain, Romania, and Australia) the IId subtype family dominate (Díaz et al., 2015; Imre et al., 2013; Quilez et al., 2008; Yang et al., 2014).

The predominant IIaA15G2R1 subtype has previously been reported as the most prevalent subtype 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, 2010a), including France (Follet et al., 2011; Rieux et al., 2014, 2013c, 2013a), thus highlighting the zoonotic potential of lamb 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 is only isolated from mixed-species breeding, or whether subtype predominance is due to one restricted available host.

The IIaA16G3R1 *C. parvum* subtype has been identified in many studies of calves from France (Follet et al., 2011; Razakandrainibe et al., 2018; Rieux et al., 2013a), and in ruminants (calves, lambs, and goat kids) from other locations (Spain, Korea, Australia, and Algeria) (Díaz et al., 2015; Lee et al., 2016; Nolan et al., 2009; Sahraoui et al., 2019). In addition, human *Cryptosporidium* infections, including subtype IIaA16G3R1, have been reported in patients from Denmark and Iran (Kiani et al., 2017; Stensvold et al., 2015).

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

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

Conclusion

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

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

Mohamed Mammeri is the grateful recipient of a Cifre (Industrial Research Training Agreement) grant. He would like to thank Phileo by Lesaffre (France) and the ANRT (National Association for Technical Research), Ministry of Research (France).

The authors would like to thank all the private veterinarians for their participation in sampling: Dr J. Visse, Dr P. Autef, Dr N. Emeriau, and Dr G. Vautrain.

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Figures

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

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

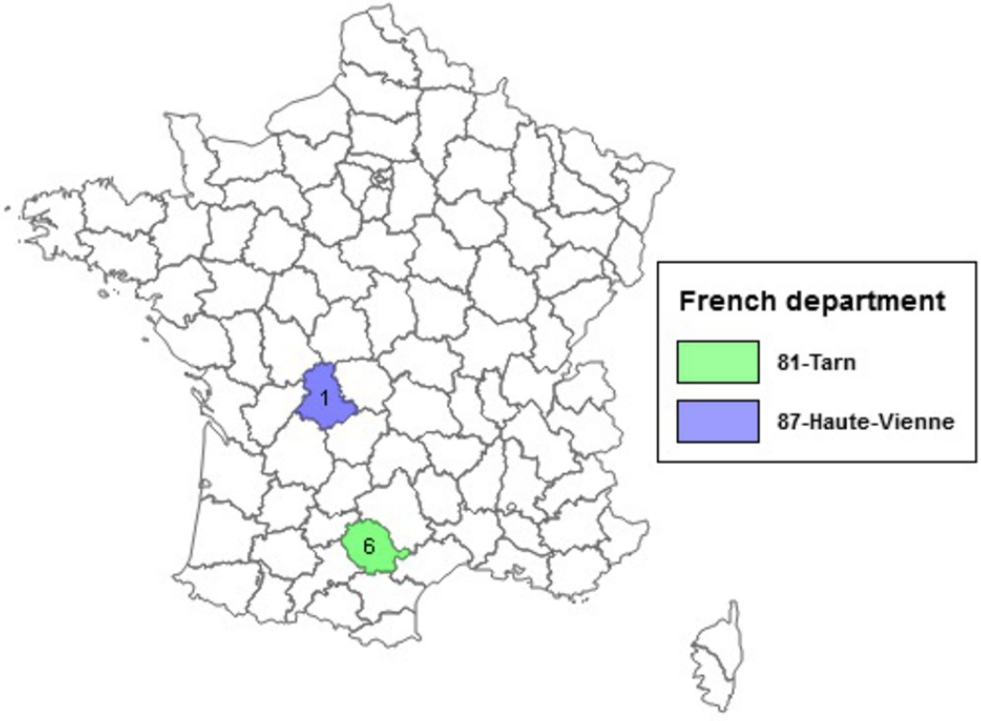
French department
81-Tarn
87-Haute-Vienne
1
6

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	*C. parvum* subtype	Accession numbers
O1	81 (Tarn)	F1 (1)	**10**	2 x 10^3	IIaA15G2R1	**MN37849**
O2		F2 (1)	10	8 x 10^4	IIaA15G2R1	**MN37850**
O12		F4 (1)	7	2 x 10^7	IIaA16G3R1	**MN37860**
O13		F5 (5)	8	1 x 10^6	IIaA15G2R1	**MN37861**
O14			8	8 x 10^6		**MN37862**
O15			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		F7 (5)	5	9 x 10^7	IIaA15G2R1	**MN37867**
O20			5	3 x 10^6		**MN37868**
O21			10	7 x 10^6		**MN37869**
O22			8	3 x 10^6		**MN37870**
O23			6	1 x 10^7		**MN37871**
O3	87 (Haute-Vienne)	F3 (9)	**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			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**