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Molecular characterization of zoonotic *Cryptosporidium* spp and *Giardia duodenalis* pathogens in Algerian sheep

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Highlights

- Detection of *Cryptosporidium* spp. and *Giardia duodenalis* in Algerian lambs using molecular tools.
- In lambs, *C. parvum, C. ubiquitum*, and *Giardia duodenalis* were identified.
- In lambs, subtypes identified were *C. parvum* IIdA16G1, IIaA21G2R1, and IIaA13G2R1.
- Assemblage E, D, and mixed Assemblage E + A of *Giardia duodenalis* were detected.
- Sheep might be a source for zoonotic *C. parvum* and *G. duodenalis* in Algeria.

Abstract

Little is known about the presence of *Cryptosporidium* spp. and *Giardia duodenalis* in Algerian sheep, nor their potential role as zoonotic reservoirs. This study aimed to investigate the occurrence and distribution of these two protists in lambs. A total of 83 fecal samples were collected from lambs (< 40 days old) from 14 different farms. Samples were screened for *Cryptosporidium* spp. and *Giardia duodenalis* presence using immunofluorescent techniques (IF). Nested PCR of the small subunit ribosomal RNA (rRNA) gene, followed by restriction fragment length polymorphism (PCR-RFLP) and sequence analyses were used to identify *Cryptosporidium* species. *C. parvum* was further subtyped by sequencing the highly polymorphic 60 kDa glycoprotein (*gp60*) gene. For *G. duodenalis*, nested PCR of the glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tpi*) genes was performed and then PCR-RFLP was used to identify *G. duodenalis* assemblages. *Cryptosporidium* oocysts and *Giardia* cysts were detected in 36/83 (43%) and 23/83 (28%) of fecal samples, respectively. Of the 21/36 (58%) *Cryptosporidium* samples that were positive with IF, 16/21 (76%) were identified as *C. parvum*, and 5/21 (24%) as *C. ubiquitum*. From 15 *C. parvum* isolates, 2 subtypes were identified within the IIa subtype family, including IIaA21G2R1 (3/15) and IIaA13G2R1 (1/15), while IIdA16G1 (11/15) was the only subtype identified from the IId subtype family. Of the 16/23 (69%) *G. duodenalis* IF-positive samples, the most frequent assemblage was ruminant-specific assemblage E (10/16), followed by assemblage D (4/16), and A + E mixed assemblages (2/16). This study is the first to identify and genotype both *Cryptosporidium* spp. and *Giardia duodenalis* in Algerian lambs, and is also the first to describe *G. duodenalis* assemblage D in small ruminants. The presence of zoonotic *C. parvum* subtype families (IIa, IId), *C. ubiquitum,* as well as *G. duodenalis* assemblage A+E, indicates that sheep could play an important role as a potential reservoir for protists.

Keywords: *Cryptosporidium; Giardia;* sheep; Algeria; genotyping; zoonosis.

1. Introduction

Cryptosporidium spp. and *Giardia duodenalis* are common zoonotic enteric protists causing clinical and subclinical infections in farm animals worldwide, and also pose a significant threat to public health (de Graaf et al., 1999).

Clinical symptoms of *Cryptosporidium* infection in small ruminants (lambs and kids) include diarrhea and weight loss, which can be fatal. This not only severely impacts small ruminant farming economies, but also creates a significant transmission risk to humans (de Graaf et al., 1999). Thus far, seven *Cryptosporidium* species have been isolated from sheep feces, including *C. parvum*, *C. ubiquitum*, *C. xiaoi*, *C. hominis*, *C. andersoni*, *C. fayeri*, and *C. suis* (Paraud and Chartier, 2012).

In Algeria, sheep and goat populations are currently estimated at 28 and 4.9 million head respectively, while the cattle population is estimated at only 1.9 million head (Ministry of Agriculture and the rural development, 2016). However very little is known about which specific *Cryptosporidium* species/subtypes infect small ruminants in Algeria. Thus far, only a few studies have characterized *Cryptosporidium* at a molecular level in calves (Baroudi et al., 2017; Benhouda et al., 2017; Ouakli et al., 2018), and only one recent molecular *Cryptosporidium* study on sheep isolates exists (Baroudi et al., 2018). On the other hand, *Giardia duodenalis* is known to infect numerous mammalian species and consists of at least eight distinct genetic groups or assemblages (A to H), often with different host specificities: assemblage A and B in humans, primates and other mammals; assemblage C and D in dogs and other canids; assemblage E in hoofed livestock; assemblage F in cats; assemblage G in rodents; and assemblage H has been reported in seals and a gull (Ballweber et al., 2010; Ryan and Cacciò, 2013). In Algeria, *G. duodenalis* was recently identified in calves, including

ruminant-specific assemblage E and zoonotic assemblage A (Baroudi et al., 2017), but no data exist for small ruminants.

The farming of small ruminants is one of the main sources of meat production in Algeria and plays a vital role in food security. As stated before, there are more than 32.9 million small ruminants in Algeria; thus farming these animals can improve the living standards of farmers and households, as well as increase the general availability of animal protein for consumption, thus helping to alleviate poverty.

Little is known about the presence of *Cryptosporidium* spp. and *Giardia duodenalis* in sheep, nor the role that these animals may play as reservoirs for these parasites. Therefore, the present work aimed to identify *Cryptosporidium* and *Giardia* at a molecular level in lambs from different northern Algerian regions.

2. Materials and Methods

Specimen collection

Between November 2015 to March 2017, 83 randomly selected lamb rectal fecal samples were collected from 14 farms across four northern Algerian provinces located in the North-Center (Djelfa and Msila), North-West (Sidi Bel Abbès), and North-East (Souk Ahras) (Fig. 1).

The Algerian sheep population is estimated at approximately 28 million head. Sheep farming mainly occurs in northern Algeria as the southern regions (Sahara) are too arid. The sheep populations from the northern provinces included in this study represent approximately one quarter (23%) of the total national sheep population. The farms included in this study were arbitrarily designated F1 to F14, and were predominantly extensive herds (free-ranging) (9/14) where animals graze freely during the day and are housed in sheds at night. Whereas in

96 intensive herds (5/14), animals were housed in farm buildings with zero access to grazing
97 (Table 1).
98 Sampled lambs were less than 40 days old, presenting with or without diarrhea. Fecal samples
99 were individually collected from lambs in plastic boxes, and were then preserved by diluting
100 1:1 in 5% (wt/vol) potassium dichromate as previously described (Bornay-Llinares et al.,
101 1999), and conserved at 4°C until use.
102 **Sample processing**
103 All samples were concentrated from 1 g of original fecal matter as previously described
104 (Castro-Hermida et al., 2005), then screened for the presence of *Giardia* cysts and/or
105 *Cryptosporidium* oocysts by direct immunofluorescence assays (IFA) (MeriFluor®
106 *Cryptosporidium*/*Giardia*, Meridian Bioscience Europe, Milano, Italy). Briefly, oocysts and
107 cysts were resuspended in 500 µL of PBS (phosphate buffered saline), then IFA was
108 performed in duplicate using 20 µL of this solution. Entire slides were examined under a
109 fluorescent microscope at 400× magnification. Samples were considered to be positive when
110 at least one *Cryptosporidium* oocyst or *Giardia* cyst was observed per slide.
111 **DNA extraction and PCR amplification**
112 Samples with positive IFA results for either parasite then underwent genomic DNA extraction
113 using the QIAamp Mini Kit (Qiagen), according to manufacturer's instructions. To disrupt
114 (oo)cyst walls, an initial step of six freeze-thaw cycles (freezing in liquid nitrogen for 5 min
115 and thawing at 95 °C for 5 min) was incorporated into the protocol.
116 To detect *Cryptosporidium* spp. in *Cryptosporidium* IFA-positive samples, nested PCR was
117 used to amplify an 830 bp fragment of the 18S SSU-rRNA gene as previously described
118 (Xiao et al., 1999). PCR products were analyzed in 2% agarose gel stained with ethidium
119 bromide (0.5 µg/mL).

To confirm the presence of *G. duodenalis* in *Giardia* IFA-positive samples, semi-nested PCR was used to amplify the glutamate dehydrogenase (*gdh*) gene and triose phosphate isomerase (*tpi*) gene. (Read et al., 2004). Amplification of a 530 bp *tpi* gene fragment was performed as previously described (Sulaiman et al., 2003). Reactions were then visualized on ethidium bromide-stained (0.5 µg/mL) 2% agarose gels.

PCR-RFLP

In order to identify *Cryptosporidium* species, positive 18S SSU-rRNA products were subjected to PCR-RFLP analysis using two endonucleases; SspI and MboII (New England BioLabs, France) as previously described (Feng et al., 2007). Digestion products were separated on 3% MetaPhor agarose (Ozyme, France). The different *Cryptosporidium* species were identified according to previously described restriction patterns (Feng et al., 2007).

For *G. duodenalis*, positive *gdh* PCR products were digested with *Nla*IV (New England Biolabs), and *tpi* PCR products with *Dde*I (New England Biolabs). RFLP analysis to determine the assemblage was directly carried out on PCR products in a 20 µL reaction volume including 10 µL of unpurified PCR product, 7.6 µL sterile water, 0.4 µL restriction enzyme, and 2 µL 10X restriction enzyme buffer. Digestions were incubated at 37°C for 3 or 4 h, for *gdh* and *tpi* respectively. Restricted fragments were separated and visualized by electrophoresis on 2% high-resolution grade agarose gel (MetaPhor) stained with ethidium bromide (0.5 µg/mL). A 50 bp DNA ladder (GeneRuler™, Thermo Scientific™) was used as a size marker. The genetic assemblages were differentiated according to previously described restriction patterns (Read et al., 2004; Sulaiman et al., 2003).

***gp60* gene analysis for *C. parvum* subtyping**

C. parvum samples were subtyped by nested PCR-sequence analysis of the 60 kDa glycoprotein locus (*gp60*), and all positive isolates were sequenced as previously described

(Gatei et al., 2006). Briefly, the PCR products were sent to Genoscreen (Lille, France), and sequenced in both directions. Consensus sequences were obtained using BioEdit software (version 5.0.6). The *C. parvum* subtypes were named using the recommended nomenclature system (Sulaiman et al., 2005; Xiao, 2010). The nucleotide sequences obtained from 15 isolates were deposed to GeneBank database under access number from : MK453405 to MK453419.

3. Results

Results from this study are summarized in Table 1.

3.1. *Cryptosporidium* species and subtype occurrence according to age and diarrhea status

Cryptosporidium spp. were detected by IFA in 36/83 (43%) of fecal samples and of which 21/36 (58%) generated positive ribosomal RNA PCRs. The majority of positive samples originated from lambs presenting with diarrhea (19/21) who were between 8 and 21 days of age. PCR RFLP sequence analysis confirmed the presence of two *Cryptosporidium* species in lambs, including *C. parvum* in 16/21 (76%) samples from all studied northern Algerian provinces, and *C. ubiquitum* in 5/21 (24%) specimens from northwestern Algeria (the Sidi Bel Abbes province only). Three *C. parvum* subtypes were identified with *gp60* gene analysis: IIdA16G1 (n = 11), IIaA13G2R1 (n = 1), and IIaA21G2R1 (n = 3) (Table 1).

3.2. *Giardia duodenalis* assemblage occurrence according to age and diarrhea status

G. duodenalis were detected by IFA in 28% (23/83) of fecal samples, of which 69% (16/23) were positive via semi-nested PCR. Three *G. duodenalis* assemblages were then identified: the ruminant-specific assemblage E (10/16); assemblage A which is infectious for humans and a number of other mammals (livestock, dogs, cats…) (2/16); and assemblage D which has been reported to infect dogs and other canids (4/16). Mixed assemblage A and E infections

were identified in two lambs (2/16). The majority of *gdh* or *tpi* PCR products identified mono-infections with ruminant-specific assemblage E. These three assemblages were mainly from non-diarrheic lamb samples (9/16), and were found at a higher rate in older lambs (>21 days) than in younger lambs (<21 days).

4. Discussion

Cryptosporidium and *Giardia* species are well-known pathogens of both domesticated farm and companion animals and are thus a significant threat to public health. There is considerable genetic diversity within both *Cryptosporidium* and *Giardia duodenalis*, as 14 *Cryptosporidium* species with several different subtypes, and 6 *Giardia* species with at least 8 *G. duodenalis* assemblages have been described (Cacciò et al., 2005). However, little is known about *Cryptosporidium* and *Giardia* occurrence rates in small ruminants in Algeria.

This is the first study to identify and perform molecular characterization of *Cryptosporidium* spp. and *G. duodenalis* in Algerian lambs, and our analysis revealed a high diversity of *Cryptosporidium* species and *G. duodenlis* assemblages within these farm animals.

In this study, IFA was used to screen for the presence of *Cryptosporidium* oocysts and *Giardia* cysts prior to performing PCR. In our study, false negative PCRs occurred, indicating that PCR sensitivity was potentially reduced, which could be due to naturally-occuring PCR inhibitors in fecal samples (Yu et al., 2009).

In this study, we report that 27 samples from diarrheal animals were positive for *Cryptosporidium*, and that 10 diarrheal animal samples were positive for *Giardia* by immunofluorescence. Most of the cryptosporidiosis-positive samples were collected from young diarrheic lambs, while the majority of *Giardia*-positive samples were from asymptomatic older animals (Robertson, 2009). This could be due to the fact that *Cryptosporidium* is a neonatal diarrhea agent, whereas *Giardia* often infects older animals

192 with subclinical symptoms. It must be noted that neonatal diarrhea is not necessarily due to
193 *Cryptosporidium* presence, as other diarrhea-causing pathogens (salmonella, viruses,
194 coccidia...) were not investigated in this study.

195 The two *Cryptosporidium* species (*C. parvum* and *C. ubiquitum*) identified in the present
196 study, have previously been reported in small ruminants from Algeria (Baroudi et al., 2018)
197 and from other countries (Paraud and Chartier, 2012). In this work, *C. parvum* was the
198 dominant species (in 16/21 animals, compared to 5/21 with *C. ubiquitum*), comparable to
199 previous small ruminant data (Drumo et al., 2012; Goma et al., 2007; Maurya et al., 2013;
200 Mueller-Doblies et al., 2008; Quilez et al., 2008; Tzanidakis et al., 2014). The *C. xiaoi* species
201 was not identified in lambs in the current study, even though it was recently reported to be
202 frequent in small ruminants from Algeria (Baroudi et al., 2018) and other locations (African
203 countries, Asian countries, and some European countries such as Norway and Poland)
204 (Kaupke et al., 2017; Parsons et al., 2015; Peng et al., 2016; Robertson, 2009).

205 In this study, the dominant *C. parvum* isolate subtype present in the lambs was IIdA16G1
206 (n = 11/16), while subtypes IIaA21G2R1 (n = 3/16) and IIaA13G2R1 (n =1/16) were reported
207 at lower rates. Our results are consistent with multiple other studies, where the *C. parvum* IId
208 subtype family is dominant in countries such as Spain, Romania, and Australia (Díaz et al.,
209 2015; Imre et al., 2013; Quilez et al., 2008; Yang et al., 2014). However in other countries
210 (the UK, Poland, New Guinea) IIa subtype families are dominant (Connelly et al., 2013;
211 Kaupke et al., 2017; Koinari et al., 2014). The identified subtypes pose a real risk to public
212 health, as the IIdA16G1 *C. parvum* subtype was recently identified in calves and human
213 children from rural regions of northern Tunisia near Algerian borders (Rahmouni et al., 2014),
214 and human *Cryptosporidium* infections—including subtype IIaA13G2R1—have been

reported in immunosuppressed individuals from Malaysia and Ethiopia (Adamu et al., 2014; Iqbal et al., 2012).

The *G. duodenalis* assemblages identified in this study indicated that lambs mainly carried *G. duodenalis* assemblage E mono-infections (10/16), which is usually found in hoofed animals, including cattle and small ruminants (Geurden et al., 2008; Paz e Silva et al., 2014; Tzanidakis et al., 2014). In addition, mixed assemblage A + E infections were identified in two lambs (2/16), similar to reports of zoonotic A + B assemblages in the USA and Australia (Santín et al., 2007; Yang et al., 2014).

Interestingly, and to the best of our knowledge, this is the first description of assemblage D in lambs, which has previously only been identified in dog or canids (Thompson, 2004). This assemblage was isolated from a single extensively-farmed herd where working dogs, stray dogs, and other wild canids are numerous. These animals could contaminate the environment (water and pasture) with *Giardia* cysts, and thus indirectly contaminate the lambs. Of note, it has been reported that intensive contact between dogs and other animals (pigs and other wild animals) could lead to assemblage D transmission (Ryan and Cacciò, 2013; Sprong et al., 2009). Other large-scale studies are needed to better understand *Giardia* circulation, particularly of those assemblages not well adapted to ruminants.

Zoonotic pathogenic *C. parvum* and *G. duodenalis* subtypes are reported to be common in dairy farm calves from the Algiers region (Baroudi et al., 2017). Our results suggest that lambs may thus be an important reservoir for *G. duodenalis* in Algeria. Further investigations are required to determine whether this observation holds true in other parts of the country, preferably with larger sample sizes to better understand the epidemiology of cryptosporidiosis and giardiosis in lambs.

In conclusion, the present work shows that *C. parvum*, *C. ubiquitum*, and *Giardia duodenalis* commonly occur in Algerian lambs, and that these data strongly suggest that sheep may be an important reservoir of zoonotic *C. parvum* and *Giardia duodenalis* in Algeria. However, further research is necessary to characterize the prevalence of various *Cryptosporidium* species and *Giardia duodenalis* assemblages in young lambs, goat kids, and calves, and also in other hosts such as humans, to fully understand the transmission dynamics of these protists in Algeria.

This is the first report of both *Cryptosporidium* spp. and *G. duodenalis* infections in Algerian lambs, and could serve as baseline data for further investigations to better understand cryptosporidiosis and giardiosis epidemiology in Algeria.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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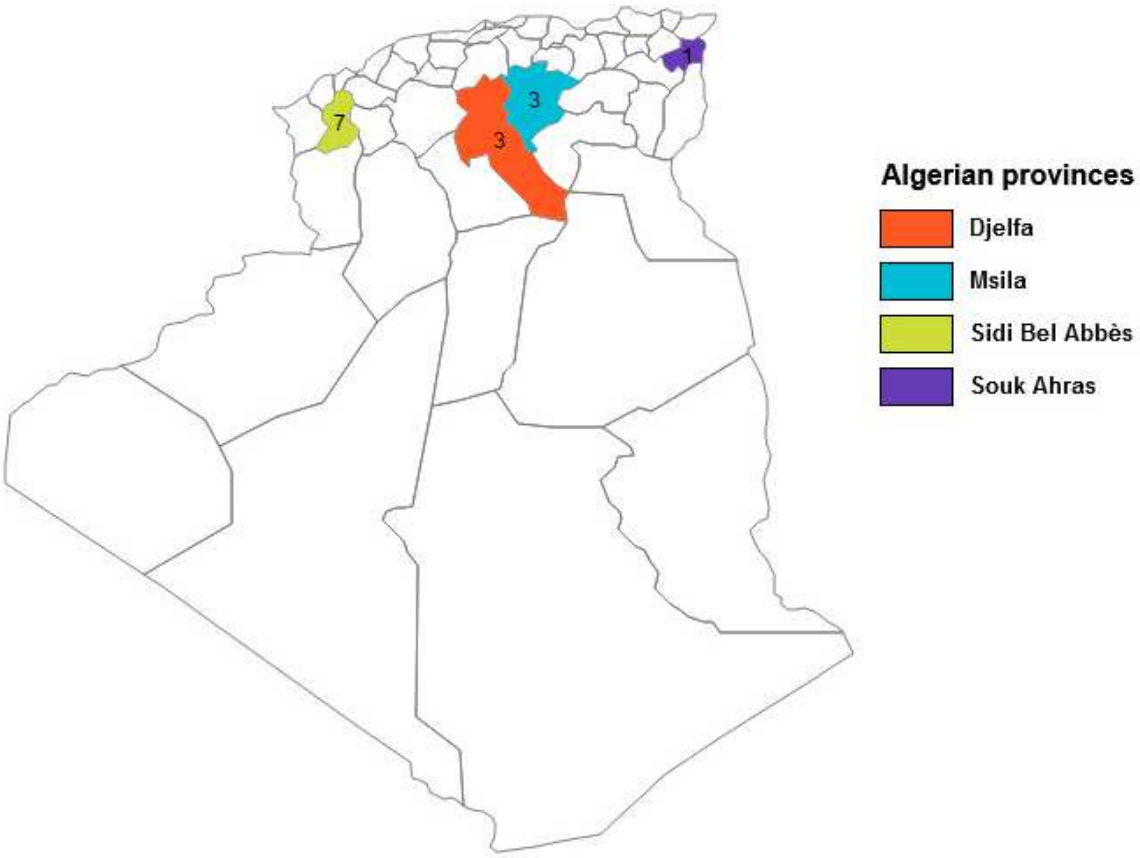
Figure 1. **Map of Algeria indicating the location, geographical distribution, and number of sheep farms investigated in this study (Djelfa, Msila, Sidi Bel Abbès, and Souk Ahras provinces).**



Tables

Table 1. Occurrence of *Cryptosporidium* species and Giardia assemblages in lambs from Djelfa, Msila, Sidi Bel Abbès and Souk Ahras provinces, Algeria.

Farm locations	No. of farms	Extensive farms	Intensive farms	No. of samples tested	*Cryptosporidium spp.*					*Giardia duodenalis*			
					No. of *Cryptosporidium* positive samples via IF (Farms indicated)	No. of IFA-positive samples from diarrheic lambs	No. of *Cryptosporidium*-positive samples via PCR	*Cryptosporidium* species	*C. parvum* subtypes (No. /Total)	No. of *Giardia positive* samples via IF (Farms indicated)	No. of IFA-positive samples from diarrheic lambs	No. of Giardia-positive samples via PCR	Giardia assemblages (No./Total)
Djelfa	3	*F9	F10, F11	24	7 (F9,F10,F11)	3	3	*C. parvum* (3)	IIaA13G2R1(1/3)	7 (F9, F11)	0	4	E (2/4)
													D (1/4)
									IIdA16G1 (2/3)				A+E (1/4)
Msila	3	F12	F13, F14	17	4 (F12,F14)	4	4	*C. parvum* (4)	IIdA16G1 (3/4)	2 (F12, F13)	0	2	E (2/2)
Sidi Bel Abbès	7	F3, F4, F5, F6, F7, F8	F2	37	22 (F2,F4,F5,F6,F7,F8)	17	11	*C. parvum* (6)	IIdA16G1 (6/6)	14 (F2, F3,F4, F5, F7, F8)	10	10	E (6/10)
													D (3/10)
								C. ubiquitum (5)	-				A+E (1/10)
Souk Ahras	1	F1	-	5	3 (F1)	3	3	*C. parvum* (3)	IIaA21G2R1(3/3)	0	0	0	-
Total	14	9	5	83	36	27	21	2 species	3 subtypes	23	10	16	3 assemblages

**F: Farms included in this study were arbitrarily designated from F1 to F14.*