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

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2 **pathogens in Algerian sheep**

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15

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.

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

73　ruminant-specific assemblage E and zoonotic assemblage A (Baroudi et al., 2017), but no data

74　exist for small ruminants.

75　The farming of small ruminants is one of the main sources of meat production in Algeria and

76　plays a vital role in food security. As stated before, there are more than 32.9 million small

77　ruminants in Algeria; thus farming these animals can improve the living standards of farmers

78　and households, as well as increase the general availability of animal protein for consumption,

79　thus helping to alleviate poverty.

80　Little is known about the presence of *Cryptosporidium* spp. and *Giardia duodenalis* in sheep,

81　nor the role that these animals may play as reservoirs for these parasites. Therefore, the

82　present work aimed to identify *Cryptosporidium* and *Giardia* at a molecular level in lambs

83　from different northern Algerian regions.

84　**2. Materials and Methods**

85　**Specimen collection**

86　Between November 2015 to March 2017, 83 randomly selected lamb rectal fecal samples

87　were collected from 14 farms across four northern Algerian provinces located in the North-

88　Center (Djelfa and Msila), North-West (Sidi Bel Abbès), and North-East (Souk Ahras) (Fig.

89　1).

90　The Algerian sheep population is estimated at approximately 28 million head. Sheep farming

91　mainly occurs in northern Algeria as the southern regions (Sahara) are too arid. The sheep

92　populations from the northern provinces included in this study represent approximately one

93　quarter (23%) of the total national sheep population. The farms included in this study were

94　arbitrarily designated F1 to F14, and were predominantly extensive herds (free-ranging)

95　(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.

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 *Cryptosporidiu*m oocyst or *Giardia* cyst was observed per slide.

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).

120   To confirm the presence of *G. duodenalis* in *Giardia* IFA-positive samples, semi-nested PCR

121   was used to amplify the glutamate dehydrogenase (*gdh*) gene and triose phosphate isomerase

122   (*tpi*) gene. (Read et al., 2004). Amplification of a 530 bp *tpi* gene fragment was performed as

123   previously described (Sulaiman et al., 2003). Reactions were then visualized on ethidium

124   bromide-stained (0.5 µg/mL) 2% agarose gels.

125   **PCR-RFLP**

126   In order to identify *Cryptosporidium* species, positive 18S SSU-rRNA products were

127   subjected to PCR-RFLP analysis using two endonucleases; SspI and MboII (New England

128   BioLabs, France) as previously described (Feng et al., 2007). Digestion products were

129   separated on 3% MetaPhor agarose (Ozyme, France). The different *Cryptosporidium* species

130   were identified according to previously described restriction patterns (Feng et al., 2007).

131   For *G. duodenalis*, positive *gdh* PCR products were digested with *Nla*IV (New England

132   Biolabs), and *tpi* PCR products with *Dde*I (New England Biolabs). RFLP analysis to

133   determine the assemblage was directly carried out on PCR products in a 20 µL reaction

134   volume including 10 µL of unpurified PCR product, 7.6 µL sterile water, 0.4 µL restriction

135   enzyme, and 2 µL 10X restriction enzyme buffer. Digestions were incubated at 37°C for 3 or

136   4 h, for *gdh* and *tpi* respectively. Restricted fragments were separated and visualized by

137   electrophoresis on 2% high-resolution grade agarose gel (MetaPhor) stained with ethidium

138   bromide (0.5 µg/mL). A 50 bp DNA ladder (GeneRuler[TM], Thermo Scientific[TM]) was used as

139   a size marker. The genetic assemblages were differentiated according to previously described

140   restriction patterns (Read et al., 2004; Sulaiman et al., 2003).

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

142   *C. parvum* samples were subtyped by nested PCR-sequence analysis of the 60 kDa

143   glycoprotein locus (*gp60*), and all positive isolates were sequenced as previously described

144 (Gatei et al., 2006). Briefly, the PCR products were sent to Genoscreen (Lille, France), and

145 sequenced in both directions. Consensus sequences were obtained using BioEdit software

146 (version 5.0.6). The *C. parvum* subtypes were named using the recommended nomenclature

147 system (Sulaiman et al., 2005; Xiao, 2010). The nucleotide sequences obtained from 15

148 isolates were deposed to GeneBank database under access number from : MK453405 to

149 MK453419.

150 **3. Results**

151 Results from this study are summarized in Table 1.

152 **3.1. *Cryptosporidium* species and subtype occurrence according to age and diarrhea**

153 **status**

154 *Cryptosporidium* spp. were detected by IFA in 36/83 (43%) of fecal samples and of which

155 21/36 (58%) generated positive ribosomal RNA PCRs. The majority of positive samples

156 originated from lambs presenting with diarrhea (19/21) who were between 8 and 21 days of

157 age. PCR RFLP sequence analysis confirmed the presence of two *Cryptosporidium* species in

158 lambs, including *C. parvum* in 16/21 (76%) samples from all studied northern Algerian

159 provinces, and *C. ubiquitum* in 5/21 (24%) specimens from northwestern Algeria (the Sidi Bel

160 Abbes province only). Three *C. parvum* subtypes were identified with *gp60* gene analysis:

161 IIdA16G1 (n = 11), IIaA13G2R1 (n = 1), and IIaA21G2R1 (n = 3) (Table 1).

162 **3.2. *Giardia duodenalis* assemblage occurrence according to age and diarrhea status**

163 *G. duodenalis* were detected by IFA in 28% (23/83) of fecal samples, of which 69% (16/23)

164 were positive via semi-nested PCR. Three *G. duodenalis* assemblages were then identified:

165 the ruminant-specific assemblage E (10/16); assemblage A which is infectious for humans

166 and a number of other mammals (livestock, dogs, cats…) (2/16); and assemblage D which has

167 been reported to infect dogs and other canids (4/16). Mixed assemblage A and E infections

168 were identified in two lambs (2/16). The majority of *gdh* or *tpi* PCR products identified

169 mono-infections with ruminant-specific assemblage E. These three assemblages were mainly

170 from non-diarrheic lamb samples (9/16), and were found at a higher rate in older lambs (>21

171 days) than in younger lambs (<21 days).

4. Discussion

173 *Cryptosporidium* and *Giardia* species are well-known pathogens of both domesticated farm

174 and companion animals and are thus a significant threat to public health. There is

175 considerable genetic diversity within both *Cryptosporidium* and *Giardia duodenalis*, as 14

176 *Cryptosporidium* species with several different subtypes, and 6 *Giardia* species with at least 8

177 *G. duodenalis* assemblages have been described (Cacciò et al., 2005). However, little is

178 known about *Cryptosporidium* and *Giardia* occurrence rates in small ruminants in Algeria.

179 This is the first study to identify and perform molecular characterization of *Cryptosporidium*

180 spp. and *G. duodenalis* in Algerian lambs, and our analysis revealed a high diversity of

181 *Cryptosporidium* species and *G. duodenlis* assemblages within these farm animals.

182 In this study, IFA was used to screen for the presence of *Cryptosporidium* oocysts and

183 *Giardia* cysts prior to performing PCR. In our study, false negative PCRs occurred, indicating

184 that PCR sensitivity was potentially reduced, which could be due to naturally-occuring PCR

185 inhibitors in fecal samples (Yu et al., 2009).

186 In this study, we report that 27 samples from diarrheal animals were positive for

187 *Cryptosporidium*, and that 10 diarrheal animal samples were positive for *Giardia* by

188 immunofluorescence. Most of the cryptosporidiosis-positive samples were collected from

189 young diarrheic lambs, while the majority of *Giardia*-positive samples were from

190 asymptomatic older animals (Robertson, 2009). This could be due to the fact that

191 *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

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

216   Iqbal et al., 2012).

217   The *G. duodenalis* assemblages identified in this study indicated that lambs mainly carried

218   *G. duodenalis* assemblage E mono-infections (10/16), which is usually found in hoofed

219   animals, including cattle and small ruminants (Geurden et al., 2008; Paz e Silva et al., 2014;

220   Tzanidakis et al., 2014). In addition, mixed assemblage A + E infections were identified in

221   two lambs (2/16), similar to reports of zoonotic A + B assemblages in the USA and Australia

222   (Santín et al., 2007; Yang et al., 2014).

223   Interestingly, and to the best of our knowledge, this is the first description of assemblage D in

224   lambs, which has previously only been identified in dog or canids (Thompson, 2004). This

225   assemblage was isolated from a single extensively-farmed herd where working dogs, stray

226   dogs, and other wild canids are numerous. These animals could contaminate the environment

227   (water and pasture) with *Giardia* cysts, and thus indirectly contaminate the lambs. Of note, it

228   has been reported that intensive contact between dogs and other animals (pigs and other wild

229   animals) could lead to assemblage D transmission (Ryan and Cacciò, 2013; Sprong et al.,

230   2009). Other large-scale studies are needed to better understand *Giardia* circulation,

231   particularly of those assemblages not well adapted to ruminants.

232   Zoonotic pathogenic *C. parvum* and *G. duodenalis* subtypes are reported to be common in

233   dairy farm calves from the Algiers region (Baroudi et al., 2017). Our results suggest that

234   lambs may thus be an important reservoir for *G. duodenalis* in Algeria. Further investigations

235   are required to determine whether this observation holds true in other parts of the country,

236   preferably with larger sample sizes to better understand the epidemiology of cryptosporidiosis

237   and giardiosis in lambs.

238 In conclusion, the present work shows that *C. parvum*, *C. ubiquitum*, and *Giardia duodenalis*

239 commonly occur in Algerian lambs, and that these data strongly suggest that sheep may be an

240 important reservoir of zoonotic *C. parvum* and *Giardia duodenalis* in Algeria. However,

241 further research is necessary to characterize the prevalence of various *Cryptosporidium*

242 species and *Giardia duodenalis* assemblages in young lambs, goat kids, and calves, and also

243 in other hosts such as humans, to fully understand the transmission dynamics of these protists

244 in Algeria.

245 This is the first report of both *Cryptosporidium* spp. and *G. duodenalis* infections in Algerian

246 lambs, and could serve as baseline data for further investigations to better understand

247 cryptosporidiosis and giardiosis epidemiology in Algeria.

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253 **Conflict of interest**

254 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).
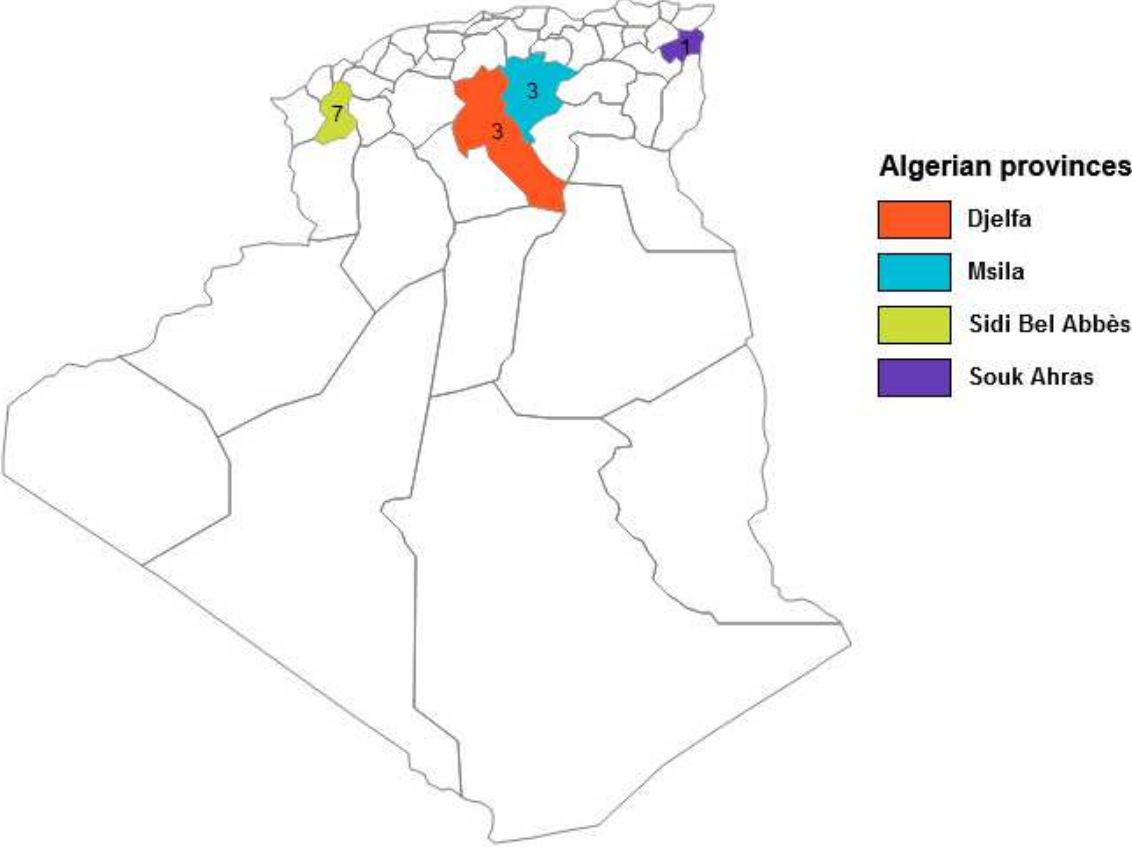
Tables

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) / IIdA16G1 (2/3)	7 (F9, F11)	0	4	E (2/4) / D (1/4) / 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) / *C. ubiquitum* (5)	IIdA16G1 (6/6) / -	14 (F2, F3,F4, F5, F7, F8)	10	10	E (6/10) / D (3/10) / 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.