Original Article

Molecular identification of *Cryptosporidium* spp. isolated from dairy calves in the Northern region of Algeria

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Abstract

Cryptosporidium spp. is the most frequent agent in Algeria that causes neonatal diarrhea in calves, with high symptomatic diarrhea in pre-weaned dairy calves. The study aims to measure the prevalence, distribution, and genotype of *Cryptosporidium*-infected calves in one of Algeria's high milk production areas (North Central and Northeast). From 35 dairy cattle farms were selected 172 (77 with diarrheic and 95 without diarrheic symptoms) calves aged less than 60 days. Twenty-three farms (65.71%) were detected with cryptosporidiosis using a modified Ziehl-Neelsen stain, whereby 68 calves (39.53%) were obtained positive. The Copro-ELISA test gave an individual prevalence of (37.66%, 22.11%) in calves with diarrhea and asymptomatic calves, respectively. Fifty positive cases confirmed with the previous tests reconfirmed by the target of *S18* gene (*SSU rRNA*), in addition to the sequencing, were obtained from species *C. parvum* in all the analyzed samples. The subgenotype of *C. parvum* was obtained by amplifying the target gene of *gp60*, successfully confirmed by *C. parvum* type II (IIaA15G2R, IIaA16G2R1, and IIaA17G2R1). Our results confirmed the frequency of cryptosporidiosis in dairy calves from farms in Northern Algeria, with a pathogenic role in the calves, with potential zoonotic risk from food contamination.

Keywords: Algeria, calves, *Cryptosporidium* spp., diarrhea, ELISA, PCR

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Introduction

Cryptosporidiosis is a digestive infestation disease caused by an apicomplexan protozoan of the genus *Cryptosporidium* (Xiao *et al*., 2004). The disease is shared between humans and mammals such as cattle, especially the pre-weaned calves, which could be infected with zoonotic species such as *Cryptosporidium parvum* (Xiao *et al*., 2004; Santin., 2020). Also, Cryptosporidiosis is often associated with other germs such as *Rotavirus*, *Coronavirus*, and *E. coli* F5 in infected calves (Khelef *et al*., 2007). Neonatal diarrhea in calves under three months of age caused by *Cryptosporidium* resulted in significant economic losses related to the cost of treatment, growth retardation, and calf mortality in severe cases, such as during the mixt infections with other germs (Boussena *et al*., 2009). In humans, *Cryptosporidium* is one of the causes of the water-borne disease, with a severe digestive infestation, which can be fatal for immunocompromised patients (HIV+) (Cacciò *et al*., 2016; Xiao *et al*., 2017). In addition, it is the second causative agent of diarrhea in children after *Rotavirus* (Striepen *et al*., 2013). In Algeria, few studies have been carried out on the details of the molecular epidemiology of *Cryptosporidium*, despite that it has been detected in humans, especially in rural areas (Ouldrouis-Saoudi *et al*., 2011). Some of the studies carried out in farms from the North of Algeria show the frequency of *Cryptosporidium* spp. in pre-weaned diarrheal calves with a prevalence ranging from 16% to 39% (Akam *et al*., 2009; Ouchene *et al*., 2012). Those studies showed the distribution, prevalence, and species of *Cryptosporidium* spp. strongly depend on the calves' age, and the breeding region obtained the species *C. andersoni*, *C. bovis*, *C. ryanae* with the dominance of *C. parvum* (Benhouda *et al*., 2017; Ouakli *et al*., 2018). Given the dual health and economic significance of this enteropathogen in dairy cattle farms, particularly in Northern Algeria, the present study conducted an epidemiological survey aimed at generating comprehensive data on the parasite. Both conventional and advanced diagnostic methods, including coprological and immunoenzymatic examinations as well as molecular techniques (nested PCR followed by sequencing), were applied. The survey targeted both diarrheic and asymptomatic calves from high milk-producing farms in North Central and Northern regions of the country, intending to identify circulating species and subgenotypes, and assess their zoonotic potential.

Materials and Methods

Study area: The research involved five provinces in Northern Algeria, divided into two zones, Zone A in the North Central Algeria represented by the province of Blida, Médéa, Tizi-Ouzou, and Bouira and Zone B in Northeast of the country represented by the province of Sétif (Fig. 1). The study was carried out between January 2015 and March 2020 during the different seasons to study the influence of climatic and environmental conditions on the occurrence of the disease.

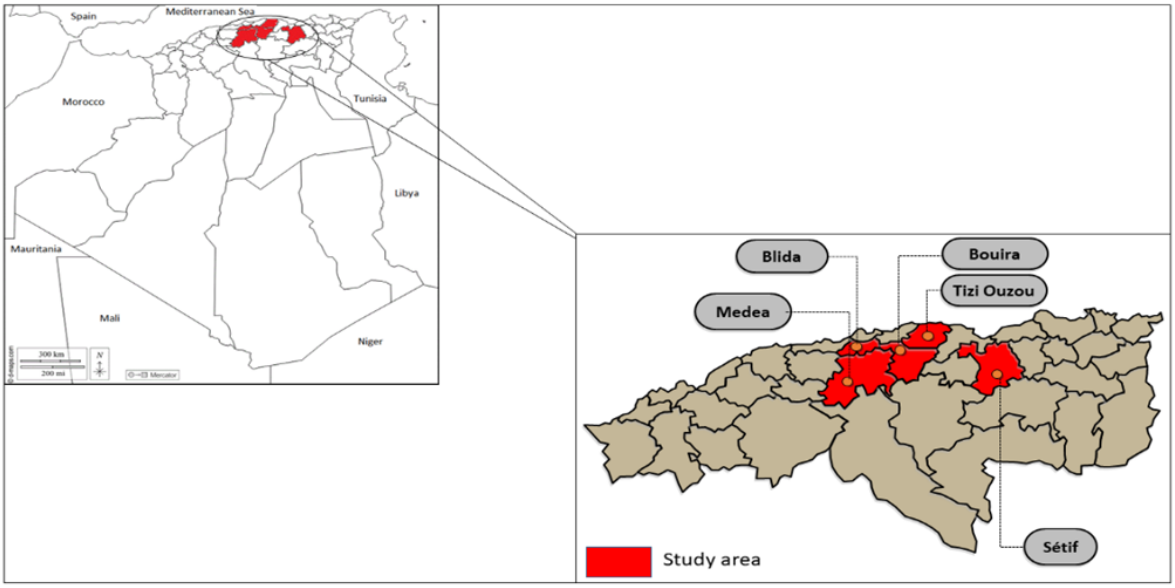


Figure 1 Geographical location of investigated areas for cryptosporidiosis in the calves from Northern of Algeria.

Samples collection: The samples were collected from 35 dairy cattle farms located in Zone A and B according to the regular customers of private veterinarians who work in this region. Each farm has a cattle size between 50 to 250 dairy cows composed mainly of the breeds: Montbeliarde, Prim'holstein, Alpine Brown, and Fleckvieh. All the samples were obtained from dairy calves less than 60 days old, and the target population was divided into six age classes from the first week of age, with an interval of one week between each category from the first week to 60 days of age. 172 fecal samples were collected, including 77 from diarrheic calves and 95 from non-diarrheic healthy calves. The collection and storage of samples started with cleaning the anal area using toilet paper and then collecting the stool from the rectum using the index finger of the

gloved hand after excitation of the rectum and keeping it in a plastic sterile stool container. Immediately, the collection containers of samples were identified and either labeled with an indelible pen and then transported in an isothermal cooler to the laboratory. Each sample was accompanied by an information sheet that essentially registered the identification of the animal, clinical condition, degree of dehydration, the calving season, and the conditions of breeding.

Laboratory analysis: At the laboratory, each sample was divided into two sterile tubes; one was treated fresh by copro examination or by preserving with potassium dichromate (Cr2K2O7) at 2.5% per ½ of sample volume to avoid contamination by molds, yeasts, and fungi, and then stored immediately in the refrigerator (4-8°C). The other tubes were frozen at -20°C without adding potassium dichromate for the copro-ELISA and molecular analysis.

Microscopy: To detect *Cryptosporidium* oocysts in the feces of all the 172 samples by concentrating the number of oocysts with the Ritchie technique simplified by Allen and Ridney (1970), followed by the Ziehl-Neelsen staining technique modified by Henriksen and Polhenz (1981).

ELISA copro-antigen test: The samples being analyzed by microscopic examination (172 samples) were analyzed by CpAg-ELISA test. According to the manufacturer's instructions, two ELISA test kits were used: a tetravalent ELISA kit for *Rotavirus*, *Coronavirus*, *E. coli* F5, and *Cryptosporidium* spp., and a monovalent ELISA kit specific to bovine *Cryptosporidium* spp. (BIO K 348 ELISA, Bio-X Diagnostics, Rochefort, Belgium).

Molecular analysis: The molecular analysis obtained for 50 positive samples confirmed by microscopy and by CpAg-ELISA were analyzed with nested PCR to target the gene (*18S SSU rRNA*) of the small subunit ribosomal ribonucleic acid gene (*SSU rRNA* ~ 480 bp) followed by nested PCR targeting the gene encoding glycoprotein 60kda (*GP60*). A commercial DNA extraction kit for fecal samples (QIAamp® DNA Stool kit, QIAGEN, Hilden, Germany) was used with 180-220 mg of each sample to extract DNA of oocysts according to the manufacturer's instructions, and the template DNA was kept at -20°C for further study. The amplification of a 480 bp fragment of the *18S gene* (*SSU rRNA*) was prepared by a 40 µL reaction mixture consisting of 40 µL of DNA, 80 µL of mixers containing Taq DNA polymerase, d NTP, reaction buffer, glycerol, bromophenol, stabilizer (HIPI Plus 5X PCR Master Mix/ELPiS), 10 µL of each primer (Xiao *et al*., 1999; Una and Rongchang., 2012) and 26 µL of distilled water. The thermal cycler of the two nested PCR reactions started with a cycle of initiation for 3 minutes at 94°C, followings by 35 cycles of three steps: denaturation for 94°C at 45 seconds, annealing for 55°C at 45 seconds, and extension for 72°C at 1 min, and the reaction ends with a final extension step of 72°C for 7 minutes. To determine the subgenotype of *C. parvum* from the positive samples were analyzed the gene encoding 60 kDa glycoprotein (*GP60*) by amplification of 840 bp using a nested PCR protocol with a reaction mixture similar to the reaction mixture used in the identification of the *18S* gene, the modifications only in the used primers (Table 1). The thermal cycler steps are identical to those followed for the amplification of the *SSU rRNA* gene. The different fragments of amplified DNA were separated by electrophoresis (85 volts for 30 minutes) on 1% agarose gel bathed in a TAE buffer (Trisbase, acetate acid, and EDTA) solution. The visualization is done with the help of a gel reader (BIO-RAD, Gel Doc XR+) linked to the microcomputer to visualize the immigration bands of the DNA fragments.

Table 1 The primers used in this study for DNA sequence and location on the *Cryptosporidium* genome.

Primer name	Primer sequence (5′→3′)	Target gene	References
CPP/F1	TTCTAGAGCTAATACATGCG	*18S*	(Xiao, 2010)
CPP/R1	CCCTAATCCTTCGAAACAGGA		
CSP/F2	GGAAGGGTTGTATTTATTAGATAAAG		
CSP/R2	AAGGAGTAAGGAACAACCTCCA		
Gp 60F1	ATG AGA TTG TCG CTC ATT ATC	*gp60*	(Xiao and Fayer, 2008)
Gp 60R1	TTA CAA CAC GAA TAA GGC TGC		
CDCGP60/F2	TCC GCT GTA TTC TCA AGC C		
CDCGP60/R2	GCA GAG GAA CCA GCA TC		

Data analysis: Sequencing samples were performed at a biomolecular company (Cosmogenetech Co, Daejeon, KOREA), and sequence analysis was performed by Geneious software, version 9.0 (Olsen, 2014). Sequenced samples were trimmed and assembled by *de novo* sequence assemblers to generate consensus sequences. NCBI was used to determine the similarity and observe the obtained sequence's phylogenetic relationships with the existing sequences in GenBank. In addition, *C. meleagridis* were added to the analysis as an outgroup species. MEGA v.6 software, the Maximum Likelihood (ML) method is used to root the phylogenetic tree. The Bayesian Information Criterion (BIC) value was determined with the HKY +I (Hasegawa-Kishino-Yano + invariant sites) method, with 1000 bootstrap replications estimated to achieve a high confidence level.

Statistical analysis: The prevalence of *Cryptosporidium* infection obtained using the different laboratory tests were analyzed by the Chi-square (χ^2) test dependence with a significance level of 5% ($P < 0.05$), to compare the infection rates in the different study regions, as well as to investigate the dependence of diarrheal infection in calves less than 60 days of age. We also used the Student *t*-test to compare the *Cryptosporidium* infection

rates in the different age groups of the target population with a significance level of 5%.

Results

Microscopy: *Cryptosporidium* spp. was detected in 23 farms out of 35 farms in the two regions (Zone A and B) with an estimated prevalence of 65.71% (95% confidence interval; [63.71% - 67.71%] (Table 2). Microscopy examination obtained 68 positive samples for *Cryptosporidium* spp. with an individual prevalence of 39.5 ± 0.5%. This prevalence was higher in diarrheic calves (59.5%) compared to the non-diarrheic calves (21%) under 30 days of age ($P < 0.05$). Also, the result showed the prevalence is higher in the North Central region than in the Northeast region ($P < 0.05$). Infected farms prevalence was estimated at 68.0% and 60.0 ± 2.0% in the North Central and Northeast regions, respectively; however, it is not statistically significant ($P = 0.9$) in the two study regions. On the other hand, the difference in the individual prevalence was 45.66 ± 0.5% in the North Central region and 22.2 0.5% in the Northeast region with the difference being statistically significant ($P = 0.01$) (Table 2).

Table 2 Farms and individual prevalence of cryptosporidiosis estimated by microscopy (modified Ziehl-Neelsen stain, 1981).

Areas	Number of farms investigated	Number of farms affected (%; CI= ± 2%)	Number of samples	Number of positive cases (%; CI = ± 0.5%)
North Central (A)	25	17(68%)	127	58(45.66%)
Northeast (B)	10	6(60%)	45	10(22.22%)
Total	35	23(65.71%)	172	68(39.50%)

Copro-ELISA test: With the ELISA test, 50 calves out of 172 (29.09%) confirmed the existence of *Cryptosporidium* spp. Also, the cases were detected by ELISA more frequently in sick calves than in symptomatic healthy calves, with respective prevalences of 37.66 ± 1.71% and 22.11 ± 0.5%. The chi-square test showed a dependence on the clinical status of the calves sampled ($P < 0.05$), which shows the pathogenic role of this protozoan in the development of neonatal diarrhea in calves, especially in the first month of age, when we detected the highest prevalence (44.44 ± 1.71%). Also, with results obtained from calves age 1-7 days and 21-30 days, the Student *t*-test shows a strong relationship between the age of the calves and the occurrence of cryptosporidiosis in the diarrheic calves ($P = 0.02$), as well as this correlation was also demonstrated in the no-diarrheic calves (Table 3).

Table 3 Prevalence of cryptosporidiosis according to age and clinical state of the calves determined by the Copro-Ag-ELISA test (n=172).

Age range	Diarrheic calves			Non-diarrheic calves		
	Number of samples	Number of positive cases	Prevalence [95% CI; ± 1.71%]	Number of samples	Number of positive cases	Prevalence [95% CI; ± 0,5%]
[1-7 days]	18	8	44.44%	22	4	18.25%
[8-15 days]	24	9	37.50%	29	7	23.95%
[16-21 days]	19	6	31.58%	22	5	22.81%
[22-30 days]	9	4	44.44%	12	3	24.63%
[31-45 days]	3	1	33.33%	5	1	20.53%
[46-60 days]	4	1	25.00%	5	1	20.53%
Total	77	29	37.66%	95	21	22.11%

Molecular analysis: The nested PCR, performed on 50 samples found positive by microscopy and ELISA, targeting the 18S (*SSU rRNA*), showed that 28 samples were positive for *C. parvum* at a prevalence of 56% (28/50). According to the study regions, their distribution did not significantly differ, 55.81% in the North Central region with 57.14% in the Northeast region ($P > 0.05$). To confirm the first PCR reaction results concerning the detection of the species by the gene *18S* randomly selected and sequenced 6 samples, and all were identified as *C. parvum* and registered in GenBank under the number (MW527373-MW527378) (Fig. 2). The distribution of cryptosporidiosis and genotypes according to age, the clinical status of the calves, study areas, and GenBank access codes are shown in Tables 4 and 5. The age of infected calves with *Cryptosporidium* spp. was identified, particularly in calves less than 15 days, with a rate of 62.5% and 66.6% in the population of the North Central and Northeast regions, respectively. The *C. parvum* infestation progressively decreases with age, practically disappearing in calves aged between 30 and 60 days in the farms of the Northeast region. A total of 28 samples were amplified with the expected size of ~480 bp of *SSU rRNA*, subsequently giving 11 isolates positive to the amplification of the *gp60* locus (39.28%).

Out of 11 samples, 7 were sequenced and identified as the subgenotypes Type II (IIa A15G2R1, IIa A16G2R1, and IIa A17G2R1) of *C. parvum* (Fig. 3). 9 of the 28 samples that responded positively to the analysis targeting the *gp60* gene were isolated from farms in the North Central region, while only 2 were isolated from farms in the Northeast region. Concerning age, 50% of the samples analyzed in the two study zones show an infection rate in calves less than 15 days of age.

Table 4 Prevalence of *Cryptosporidium* spp. in diarrheic calves according to age and area of study detected by *18S* gene (n=50).

Areas	Age range	Number of isolates (diarrhoeic calves)	PCR (+) *SSU rRNA* (%)	Species (*18S* sequencing)	Genbank adhésion number
North Central	1-15 days	16	10(62,5%)	Alg_CSP Alg_CSP	MW527374 MW527376
	15-30 days	15	8(53,3%)	Alg_CSP	MW527375
	30-60 days	12	6 (50,0%)	Alg_CSP	MW527373
Northeast	1-15 days	3	2 (66,6%)	Alg_CSP	MW527377
	15-30 days	2	2 (100%)	Alg_CSP	MW527378
	30-60 days	2	0 (0%)	/	/
Total		50	28 (56%)	06	

Table 5 Prevalence of *C. parvum* subtypes in diarrheic calves and their distribution according to age and study regions (n=28).

Areas (province)	Age ranges	Number of isolates (CSP.18S)	PCR (+) gp 60 (%)	Subgenotypes of *C.parvum* (gp60)	Genbank adhésion number
North Central **- Blida** **- Tizi-Ouzou** **- Médéa**	[1 - 15 days]	10	5 (50%)	IIa.A17G2R1 IIa.A16G2R1 IIa.A16G2R1 IIa.A17G2R1 IIa.A15G2R1	MW535310 MW535312 MW535313 MW535315 MW535316
	[15 -30 days]	8	2 (25%)	/	/
	[30 - 60 days]	6	2 (33,3%)	IIa.A16G2R1 IIa.A15G2R1	MW535311 MW535314
Northeast **- Setif**	[1 - 15 days]	2	1 (50%)	/	/
	[15 - 30 days]	2	1 (50%)	/	/
	[30 - 60 days]	0	0	/	/
Total		28	11(39.28%)	7	

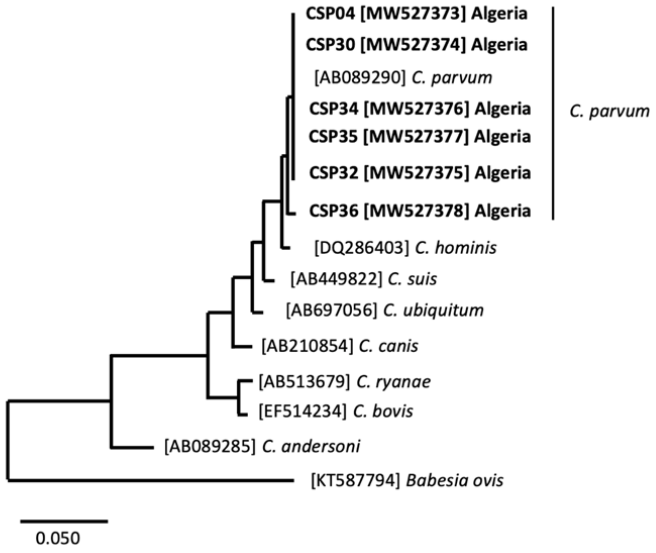


Figure 2 A phylogenetic tree based on *SSU rRNA* amplicon sequencing of *Cryptosporidium* spp. collected from Algerian calf.

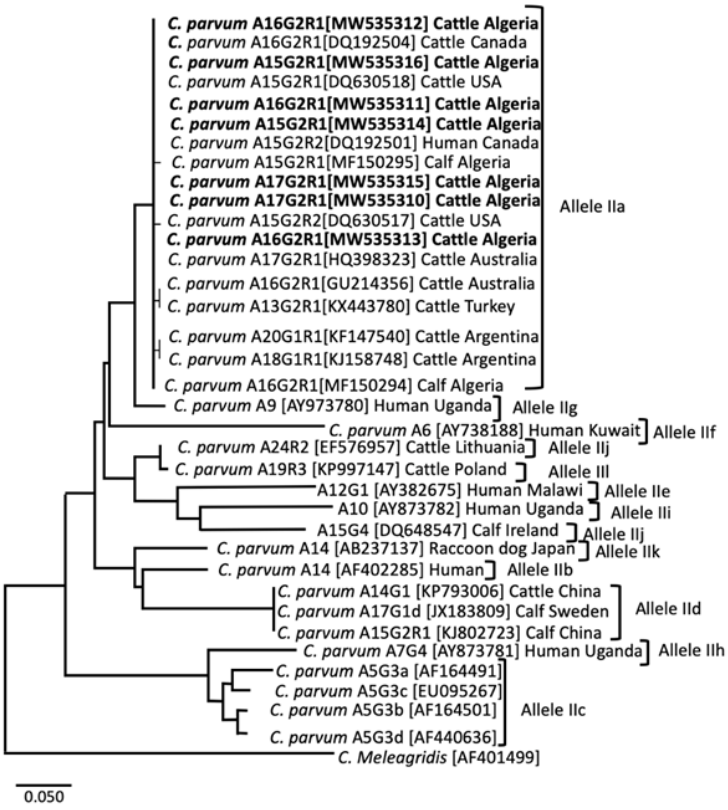


Figure 3 The evolutionary relationship between *Cryptosporidium parvum* subgenotypes at locus *gp60* isolated from calves. Bolt letters indicate samples from this study.

Discussion

Although Northern Algeria plays a significant role in dairy farming, few studies have investigated the threat posed by neonatal diarrhea caused by spp. in this region. Moreover, the available research is insufficient to provide a comprehensive understanding of the cryptosporidiosis situation in Algeria (Akam *et al*., 2009; Benhouda *et al*., 2017). Several studies mentioned that the age of calves is a risk factor that increases the susceptibility of calves to infestation by *Cryptosporidium* spp. (Millemann, 2009). In this study, it was found that impairment by *Cryptosporidium* spp. is strongly related to the age of calves. Indeed, it is common cryptosporidiosis associated with diarrhea in calves under 30 days of age (Millemann, 2009; Dadda *et al*., 2018). More precisely, in calves aged between 1 and 21 days, a period favors the infestation by *Cryptosporidium* spp. corresponding to the immune gap in young animals and their infection by other enteropathogens such as *Rotavirus*, *Coronavirus*, and the intervention of *E. coli* F5 (Bouzebda-Afri *et al*., 2007; Bouzid *et al*., 2012). It is important to note that both young and adult infected animals play a key role in contaminating the environment by shedding oocysts in their feces, thereby increasing the risk of human infection (Widmer *et al*., 2020). While previous studies have primarily linked *Cryptosporidium* transmission to symptomatic animals excreting oocysts (Paraud *et al*., 2011; Widmer *et al*., 2020), our results show that asymptomatic calves also shed oocysts, highlighting their potential role in environmental dissemination of cryptosporidiosis (Santin, 2019).

In this study, the coprological examination obtained a prevalence of 39.5%, with a detection rate of 65.7% in the investigated farms. This result is higher than that reported by Khelef *et al*. (2002), who found a prevalence of 16.7%, but this study targeted only a few farms in the North Central of Algeria. Concerning the Northeast region, a study was carried out by Ouchene *et al*. (2012) targeting farms in the region included in our study (Setif province), which gave a prevalence of 22%. Also, another investigation conducted by Akam *et al*. (2002) in the North Central region showed a significant prevalence of 33%.

The copro-ELISA test detected 50 out of 172 samples (29.09%); all of which were also positive by microscopic examination. This suggests that the remaining samples identified as positive by coprological examination may represent false positives, possibly due to misidentification of other microorganisms, such as yeasts, during microscopic observation (Casemore, 1991). In addition, investigative biases related to handling and personnel may play a role in the certainty and accuracy of laboratory results (Robinson *et al*., 2020). These results give a view of the specificity of the coprological method, which is less important than that of the ELISA test. The prevalence detected by the copro-ELISA test in this study (29.09%) was higher than that reported in previous studies 15.8% (Khelef *et al*., 2002) and 17.05% (Ouakli *et al*., 2018). In contrast, Quillet *et al*. (2006) estimated the prevalence of cryptosporidiosis in France at 32%.

Molecular analysis confirmed the infestation in 56% of the samples that were positive to the coprological ELISA test, so it was detected only the

species *C. parvum* (100%) was detected following the sequencing of the fragment of the *18S* locus. However, other research in the same regions showed the cattle of Northern Algeria are affected by other species of including the research conducted by Ouakli *et al*. (2018) that obtained the *C. bovis* with a rate of 13%, *C. andersoni* (3%), and *C. ryanae* (3%), and reported the dominance of *C. parvum* with a rate 72%. Another study conducted in the eastern region of Algeria showed the presence of *C. ryanae*, *C. bovis*, and *C. parvum* in calves less than 60 days of age (Benhouda *et al*., 2017). Baroudi *et al*. (2017) reported that the following species were detected in the North Central region (*C. bovis*, *C. andersoni*, and the most identified species was *C. parvum*). Also, in Northeast Algeria, Ouchen *et al*. (2016) found *C. parvum*, *C. bovis*, and *C. andersoni* in diarrheic and non-diarrheic calves aged of less than 60 days.

Although this study obtained only one species of *Cryptosporidium*, several subgenotypes of *C. parvum* were obtained. Also, the sequencing of the *gp60* locus fragment of 7 specimens showed the presence of subgenotypes IIa A15G2R1 in two calves, IIa A16G2R1 in three calves, and subgenotype IIa A17G2R1 in two calves. All seven specimens are from diarrheic calves belonging to farms in the North Central region. However, in the same region, Benhouda *et al*. (2017) and Ouakli *et al*. (2018) found only one subgenotype IIa A13G2R1 and IIa A16G2R1, respectively. Also, in North Central Algeria, Baroudi *et al*. (2017) have detected the subgenotype (IIa A16G2R1) identical to those found in the present study and another different subtype (IIa A17G3R1).

Although research on the molecular identification of *Cryptosporidium* in humans in Algeria is scarce, it should be noted that the subtype of *C. parvum* (IIaA15G2R1) was identified in children from Tunisia (Rahmouni *et al*., 2014), Iran (Nazemalhosseini-Mojarad *et al*., 2011), and in cattle and humans from several other countries (Feng *et al*., 2013). In addition, humans are infected mainly by sub-genotype IIa and IId (Nazemalhosseini-Mojarad *et al*., 2011); therefore, diarrheal calves shedding infesting elements represent a zoonotic potential and risk of contamination for humans in the regions included Algeria.

In conclusion, *C. parvum* infects calves from the first days of age and is responsible for neonatal diarrhea, especially in animals under 30 days. It represents an epidemiological reservoir of the zoonotic species and threatens individuals who have contact with this animal in rural areas. Further research is needed to know the relation between animal and human infection, especially at the molecular analysis level in Algeria.

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