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Epidemiology of cryptosporidiosis in dairy calves in central and eastern Algeria

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ABSTRACT: The purpose of our survey is to estimate the frequency of *Cryptosporidium* spp in dairy calves under 60 days of age, the pathogenic role of *Cryptosporidium* spp in the development of diarrhea, the intervention and association of other enteropathogens often involved in neonatal diarrhea and the possible relationship between infection and certain farming practices in order to estimate the share of risk factors, and on the other hand, to compare between the modified Ziehl-Neelsen staining method and the copro-ELISA test.

We found 35 positive samples out of 223 faecal samples analyzed by modified Ziehl-Nielsen staining, with a prevalence of 15.69%, the infected calves had 4 times the risk of diarrhea (RR= 4), the age group [8-15 days] is the most susceptible to infection compared to the other age groups (31.1%; p<0.05).

The ELISA test revealed that 33 out of 92 calves were infected by *Cryptosporidium* spp., with a prevalence of 35.87%, showing a sensitivity and specificity of Ziehl-Neelsen staining of 51.5% and 96% respectively.

Keywords: *Cryptosporidium* spp, Calf, Ziehl-Neelsen, ELISA, Colostrums, Algeria.

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INTRODUCTION

Cryptosporidium spp are ubiquitous parasites of the digestive tract of several animal species and humans (Xiao and al., 2004). These protozoa are a major cause of diarrhea in young animals (Aslanova and al., 2013). Diarrhea has been shown to be the primary cause of calf mortality in dairy cattle farms in eastern Algeria (Bouzebda and al 2007). Recent studies on dairy cattle in northern Algeria show that *Cryptosporidium* spp have been isolated more particularly from diarrhoeal calves compared to non-diarrhoeal calves (Ouakli and al., 2018, Akam and al., 2009) and the calf can become infected within the first three days of its life (Khelef and al., 2007, Bendali and al., 1999). *Cryptosporidium* causes very significant economic losses related to calf mortality and treatment costs. The importance of cryptosporidiosis is considerable both economically and zoonotic (Björkman and al., 2003, Foll and Jérôme and al., 2011).

The objectives of our study were to estimate the prevalence of *Cryptosporidium* spp. in dairy calves less than 60 days of age and their distribution by age, to assess the pathogenic role of *Cryptosporidium* spp. in the occurrence of diarrhea in young cattle, to develop hypotheses on the risk factors linked to *Cryptosporidium* spp. Infection and to evaluate the diagnostic performance of the coprological method represented by comparing it with the modified Ziehl-Neelsen stain and the copro-ELISA test.

Inadequate colostrum management, unbalanced feeding of pregnant cows, intervention by other enteropathogens and collective parking of calves of different ages appear to be the most likely risk factors associated with *Cryptosporidium* spp. infection.

MATERIALS AND METHODS

Study population

Our survey concerns dairy calves under 60 days of age, belonging to dairy cattle farms selected by lot from lists of farms in the study area, the target population is mainly composed of two breeds; Prime-Holstein, Montbeliarde. To assess the age of the calf most susceptible to cryptosporidium infection we divided the study population into six (6) age classes from the first week of age, the interval between each class is two (2) weeks to one (1) month of age and two (2) weeks from the age of 30 days to the age of 60 days.

Period and study area

Our research was carried out from March 2015 to

October 2018 in dairy cattle farms belonging to the following wilayas: Blida, Tipaza, Media (north-central Algeria), Tizi-Ouzou, and Setif (north-east Algeria).

Collection of samples

The fecal matters were collected as soon as they were released after the anal orifice was excited in sterile plastic vials, and then transported under a cold blanket in an isothermal cooler to the laboratory. We split each sample in two:

- The first, intended for coproscopy examination, treated freshly or stored under cover of 2.5% Potassium Dichromate (Cr₂K₂O₇) (1 volume/2 volume) then stored immediately in the refrigerator (4-8°C).

- The second vial was stored at a temperature of -20°C without the addition of dichromate and will be reserved for the direct ELISA test.

A first individual sheet of the information collected during the farm visit, including the number, date of sampling, age, sex, calving conditions and clinical condition of the calves, a second sheet focused on farming practices for each farm.

Laboratory analysis

At the NVS parasitological laboratory in Algiers, 223 samples (105 healthy calves and 118 sick calves) were analyzed by the modified Ziehl-Neelsen stain following treatment with the simplified Ritchie concentration technique (Starkey and al., 2006). The ELISA test was performed on 92 samples from 52 sick calves and 40 symptomatically healthy calves where we used an ELISA kit provided by the Bio-X Diagnostics laboratory, a tetravalent kit (*Rotavirus*, *Coronavirus*, *E. coli* F5 and *Cryptosporidium* spp) for the direct Copro-ELISA test. (Boussena et al., 2009)

Statistical analysis

The results were processed with the software statistica 06, are based mainly on descriptive statistics, and we used the Chi-square dependency test and the student test at the significance level $\alpha = 5\%$ for the analytical analyses.

RESULTS

Prevalence of *Cryptosporidium* spp (by Ziehl-Nielsen staining): Microscopic analysis of samples treated with modified Ziehl-Neelsen staining shows that 35 of 223 samples (Table 2) had at least

one *Cryptosporidium* spp oocyste, representing a prevalence of 15.69% (Table 1). Veal can be infected from the first days of life and the age group [8-15 days] is the most affected (31.11%) (Figure 1).

Impact of *Cryptosporidium* spp infection on the development of diarrhea in calves: *Cryptosporidi-*

um spp has been excreted in sick and non-ill calves, the prevalence of cryptosporidiosis is higher in diarrheal calves compared to non-diarrheal calves (the difference is significant ($p=0.04$) regardless of the age of the animal, with the exception of calves aged one month and calves in the age group [46-60 days] (Table 2).

Table 1: Prevalence of cryptosporidiosis according to age

Age	Number of samples	Number of positive cases	Prevalence (CI 95%)
1-7 days	45	5	11,11% [8,2%-14,03%]
[8-15days]	45	14	31,11% [28,2%-34,03%]
[16-21 days]	23	1	4,35% [1,36%-10,05%]
[22-30 days]	40	7	17,50% [14,22%-20,78%]
[31-45 days]	30	3	10,00% [5,63%-14,37%]
[46-60 days]	40	5	12,50% [9,22%-15,78%]
Total	223	35	15,69% [15,11%-16,28%]

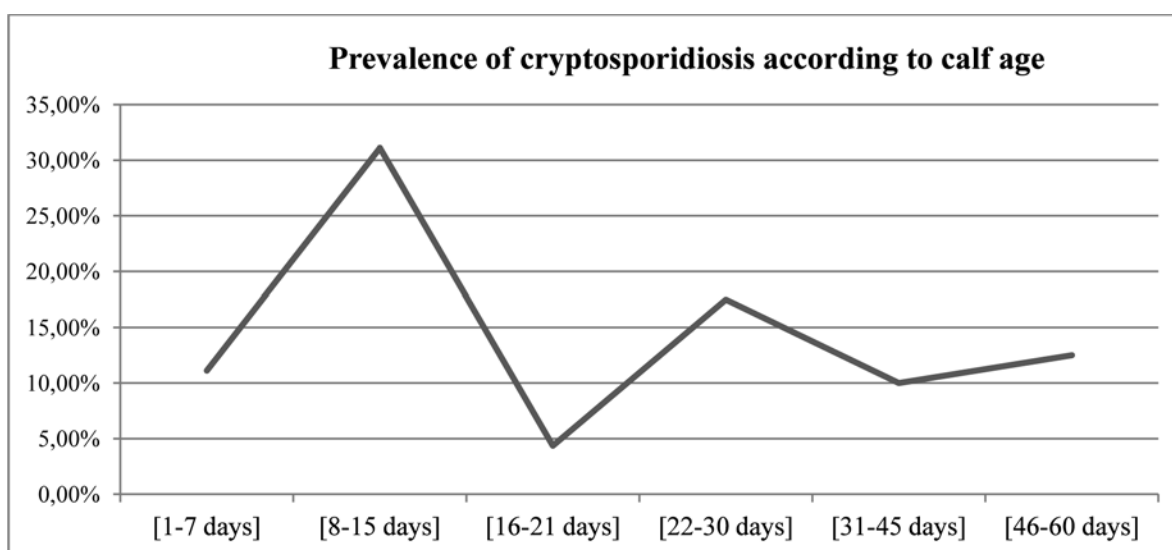


Chart 1: Prevalence of cryptosporidiosis in calves as a function of age

Table 2: Prevalence of cryptosporidiosis in diarrheal and non-diarrheal calves

Age group	D			ND		
	Number of samples	Number of positive cases	Prevalence (CI 95%)	Number of samples	Number of positive cases	Prevalence (CI%) (CI 95%)
[1-7days]	27	5	18,52% [16,71%-20,33%]	18	0	0%
[8-15days]	27	9	33,33% [31,52%-35,14]	18	5	27,78% [25,45%-30,11%]
[16-21days]	14	1	7,14% [5,33%-8,95%]	9	0	0%
[22-30days]	20	2	10,00% [8,19%-11,81%]	20	5	25% [22,67%-27,33%]
[31-45days]	16	2	12,50% [10,69%-14,31%]	14	1	7,14% [4,81%-9,47%]
[46-60days]	14	1	7,14% [5,33%-8,95%]	26	4	15,38% [13,05%-17,71%]
Total	118	20	16,95% [15,14%-18,76]	105	15	14,29% [11,96%-16,62%]

D: sampling of the sick calf (diarrhoeal stool); **ND:** sampling of non-diarrhoeal stool.

The importance of *Cryptosporidium* spp as an etiological pathogen of gastroenteritis was determined by estimating the percentage of diarrheal calf infection by *Cryptosporidium* spp in different age groups. This infection rate was compared to other rates caused by different etiological agents, whether infectious or foodborne (Table 3).

Risk factors: The analytical study showed that pregnant cow feeding, colostrum management, type of calf housing and lack of disinfection in calving facilities are the most important factors related to calf

infection by *Cryptosporidia* (Table 4).

Prevalence of cryptosporidian infection (by ELISA test): The direct ELISA test found that *Cryptosporidium* spp is the most frequently enteropathogenic in calves under 60 days of age, of which (33/92) samples were positive and had a prevalence of 35,87%; the association of other germ was found in 5 cases with *Rotavirus* in 2,17% of cases, *Coronavirus* (1%), *E. Coli* F5 (2,17% of samples examined) and the association of two viruses with *Cryptosporidium* spp was detected in one sample (1,08%) (Table 5).

Table 3: The importance of cryptosporidiosis in the etiology of neonatal diarrhea

Age class	Number of calves	Diarrheal calves		Crypto-sporid-iosis (-)	Percentage (CI95%)	RR	CI (RR)
		Crypto-sporid-iosis (+)	Percentage (CI95%)				
[1-7 days]	27	5	18,52%[16,71%-20,33%]	22	81,48%[79,67%-83,29%]	3,57	[1,87- 6,54]
[8-15days]	27	9	33,33%[31,52%-35,14%]	18	66,67%[64,86%-68,48%]	1,37	[1,6-2,7]
[16-21days]	14	1	7,14%[5,33%-8,95%]	13	92,86%[91,05%-94,67%]	4	[1,5-10,6]
[22-30days]	20	2	10,00%[8,19%-11,81%]	18	90,00%[88,19%-91,81%]	0,6	[0,09-4,05]
[31-45days]	16	2	12,50%[10,69%-14,31%]	14	87,50%[85,69%-89,31%]	2	[0,8-4,95]
[46-60days]	14	1	7,14%[5,33%-8,95%]	13	92,86%[91,05%-94,67%]	1	[0,16-6,43]
Total	118	20	16,95%[15,14%-18,76%]	98	83,05%[81,24%-84 ?86%]	2	[1, 3- 3]

RR: relative risk. (+) : presence of *Cryptosporidium* spp oocyst. (-):negative sample.

CI (RR): 95% confidence interval of RR.

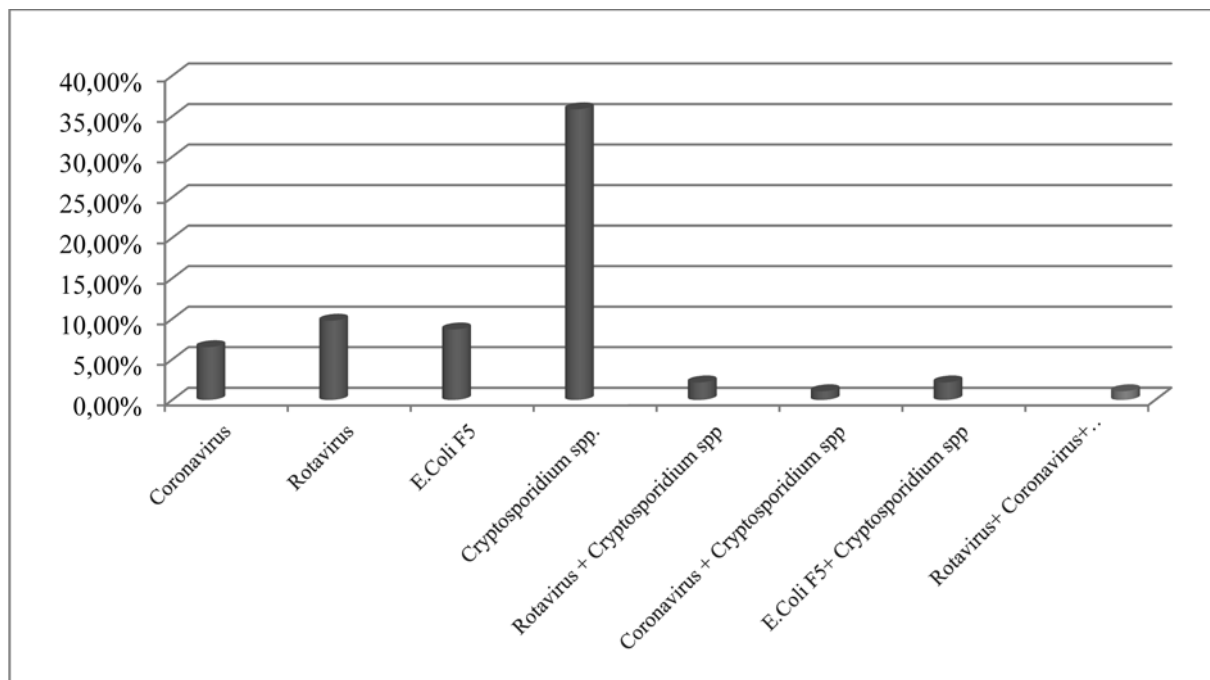
Table 4: Risk factors for *Cryptosporidium* spp

Variable	Method	(%) Crpt	χ^2 (p)
Pregnant cow ration supplements	Yes	8,02	0,01
	No	23,46	
Drying-up practice	Yes	14,51	0,01
	No	16,98	
Amount of colostrum drunk	Enough	7,4	0,04
	Inadequate	24,07	
Hygiene of the stable	Good	11,75	0,002
	Bad	19,73	
Systematic disinfection of calving rooms	Yes	13,89	0,04
	No	17,59	
Systematic disinfection of parking spaces	Yes	7,41	0,01
	No	24,07	
Type of parking	Collective	24,38	0.03
	Individual	7,1	

(%) **crpt:** prevalence of cryptosporidiosis; **p:** significance of the chi-square test (χ^2).

Table 5: Prevalence of *Cryptosporidium* spp. using tetraivalent ELISA test

Detected enteropathic agent	Number of samples (n = 92)	
	Number of positive cases	(%)
Negative sample	36	39,13%
<i>Coronavirus</i>	06	6,52%
<i>Rotavirus</i>	09	9,78%
<i>E.Coli F5</i>	08	8,70%
<i>Cryptosporidium</i> spp.	33	35,87%
<i>Rotavirus</i> + <i>Cryptosporidium</i> spp	02	2,17%
<i>Coronavirus</i> + <i>Cryptosporidium</i> spp	01	1,08%
<i>E.Coli F5</i> + <i>Cryptosporidium</i> spp	02	2,17%
<i>Rotavirus</i> + <i>Coronavirus</i> + <i>Cryptosporidium</i> spp	01	1,08%

**Figure 2:** Prevalence of the four main pathogens responsible for neonatal diarrhea in calves using the direct Copro-ELISA test

According to Figure 2, *Cryptosporidium* spp. was detected in 35% of the samples examined with a higher prevalence compared to that found by modified Ziehl-Neelsen staining (15.69%). The association of other enteropathogens was observed in 5.4% of *Cryptosporidium* spp positive cases, the other enteropathogens can be added to the factors favoring infection by this protozoan and the development of diarrhea in

calves less than 60 days of age.

Sensitivity and Specified of the modified Ziehl-Neelsen staining: To estimate the sensitivity and specificity of the Ziehl-Neelsen staining technique in the identification of *Cryptosporidium* spp oocysts in 92 samples, we used the ELISA test as a reference method and calculated the TP, FP, TN and FN to determine Sp, Se by the following formulas:

$Se = TP / TP + FN \times 100$; $Sp = TN / TN + FP \times 100$
.... (Saul Tzipori et al., 2002)

Of which:

Se: sensitivity, Sp: specificity / FP: false positive, TP: true positive

FN: false negative, TN: true negative

Out of 92 samples we detected 19 positive cases by modified Ziehl-Neelsen staining, representing a rate of 20%, this rate is lower than that found by the ELISA test (35.8%), this indicates that positive ELISA cases were not identified by Ziehl-Neelsen staining (16 false negative cases), the copro-immunological test has more specificity because the micro-cups in the ELISA test are sensitized by specific antibodies against *Cryptosporidium* spp. from Table 5 we have observed that the Ziehl-Neelsen staining method has a sensitivity of 51% and a specificity of 96% (Table 6).

DISCUSSION

Our survey was targeted at calves under 60 days of age, which represent the population at high risk and most exposed to diarrhea, the area of study is a region with a very high number of dairy livestock farms and is one of the potential areas for dairy production in Algeria. The study period included the calving seasons to explore the role of some rearing practices during calving such as animal density, calf parking and colostrum feeding as part of *Cryptosporidium* spp infection (Pwaveno et al., 2006) (Quigley et al., 2001).

In our investigation we selected the targeted population by drawing lots from the source population which was also randomly included from a general population represented by the lists of approved farms in each region to ensure the representativeness of our sample. To estimate the prevalence of cryptosporidiosis and develop hypotheses on risk factors, we used Ziehl-Neelsen staining modified by Henriksen and Pohlenz (1981) to detect *Cryptosporidium* spp oocysts in the feces of diarrheal and non-diarrheal calves, it is a reference method (Millemann et al., 2009), fast, inexpensive and

easy to read, with considerable sensitivity and specificity, more important than other coprology techniques (Khelef et al., 2002) (Aslanova et al., 2013).

The estimated prevalence of cryptosporidiosis in our survey 15.7% is similar to that reported by Khelef and al (2007) in the Mitidja area of central Algeria 16.9% (Khelef et al., 2007), in addition, another study was conducted in the same region by Akam in 2009 with a prevalence of 33% (Akam et al., 2009), similar to that found by Ouchene and al in 13 dairy cattle farms located in the Setif region in 2012 (22%) (Ouchene et al., 2012), a little higher than Björkman's result in Sweden in 2003 (14%) (Björkman et al., 2003), almost the same prevalence as that estimated for dairy calves in 5 French departments by Naciri in 1999 (17%) (Naciri et al., 1999) and lower than the prevalence in 140 farms in Vendee (France 2005) 32% (Quillet et al., 2005) our result is higher than that given by Boussena in 2008 in 14 farms in the Constantine area and one farm in the Setif area (5%) (Boussena et Sfakssi., 2009) The previously referenced surveys used the modified Ziehl-Neelsen stain.

For the prevalence determined after using the direct ELISA technique, our result (35.8%), which explains the high number of false negative samples reported in our study using Ziehl-Neelsen staining 35.8% vs 15.7%. It is higher than that reported by Bendali in the Midi-Pyrénées in France (15.6%) (Bendali and al., 1999), and the other shown by Ouakli in northern Algeria (15.8%) (Ouakli and al., 2018), by Fu Chen in China in 2012 (19%) (Fu Chen and al., 2012) and also by Follet in France (34%) (Follet et al., 2011) and is below that reported by Lise in south-western Ontario in Canada (40.5%) (Lisa A and al., 2005), these three surveys have used molecular biology methods (PCR-RFLP), this difference could be related to the husbandry practices in the populations studied as well as the conditions of each survey, or may be related to factors such as epidemiological parameters, the size and sampling mode and the laboratory diagnostic technique.

Table 6: Sensitivity and Specified of the modified Ziehl-Neelsen staining

Ziehl-Neelsen staining (n=92)	ELISA (n=92)		
	Positive	Negative	Total (%)
Positive	17	2	19 (20.6%)
Négative	16	57	73 (97,3%)
Total(%)	33 (35,8%)	59 (64,2%)	92

$Se = 17 / (16+17) \times 100 = 51,5\%$ / $Sp = 57 / (2+57) \times 100 = 96\%$

As regards risk factors, there were few analytical surveys carried out in Algeria, however, at the livestock studied in our research, we found that the correct colostrum management (quantity, quality and deadlines) significantly reduces the infection by *Cryptosporidium* spp oocysts ($p<0.05$), the colostrum quality is related to the feeding of pregnant cows and the practice of drying-up (Barwick et al., 2003), the latter two factors have been found significantly related to *Cryptosporidium* spp infection in the target population ($p<0.05$).

The presence of organic matter in the litter and moisture (especially urine) in the calf housing area and close contact between calves of different ages in the community yard were associated with an increased risk of excretion of *Cryptosporidium* spp oocysts ($p<0.05$), because these two factors ensure the survival, multiplication and transmission of the parasite from sick calves to newborns, as older calves and sick calves multiply the parasite and transmit it to particularly sensitive newborns.

Regarding the role of concomitant infections in calves, we found that 5% of the study population infested by *Cryptosporidium* spp was also affected by one of the three main enteropathogens Rotavirus, Coronavirus and *E.coli* F5, this predisposing factor requires thorough analytical studies to accurately assess its epidemiological index.

CONCLUSION

Cryptosporidiosis is a very frequent disease in dairy calves in Algeria, it may affect them from the first week of life, with a peak in excretion around 15 days of age (this moment is known as the “immune

hole”, indeed at this time colostral antibodies tend to decrease and the calf has not yet fully established its own active immunity and is therefore immune deprived and vulnerable to infection., it causes considerable economic losses due to the lack of specific treatment, it is also accompanied by high mortality in the newborn after the installation of diarrhea, where we recorded a four-fold higher risk in infected calves (RR=4).

Factors related to livestock management such as stable hygiene, calf parking irrespective of age, drying-up, feeding of pregnant cows especially in the last months of pregnancy and the intervention of other enteropathogens *Rotavirus*, *Coronavirus* and *E. coli* F5 are all major contributory factors in *Cryptosporidium* spp infection.

The copro-immunological test (ELISA-direct) reveals that *Cryptosporidium* spp is the most prevalent pathogen in the neonatal period in cattle with a significantly high frequency compared to that shown by the Ziehl-Neelsen stain modified by Henriksen and Polhenz (35% vs 16% ; $p<0.05$).

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CONFLICT OF INTEREST

None declared.

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