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Original article

Cryptosporidium-associated diarrhoea in neonatal calves in Algeria

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ABSTRACT

Neonatal calf diarrhoea triggered by the enteric protozoan parasite *Cryptosporidium* is a leading cause of morbidity and mortality in calves aged 1-month-old or younger globally. Infected cattle in general and calves in particular have also been demonstrated as major contributors of zoonotic *C. parvum* oocysts in the environment and have been linked to a number of waterborne outbreaks of human cryptosporidiosis. Little is known on the occurrence, geographical distribution, and molecular diversity of *Cryptosporidium* infections affecting bovine populations in Algeria. In this study faecal specimens were randomly collected from 460 cattle aged between two days and 18 months on 10 farms located in the provinces of Aïn Defla, Blida, Sétif, and Tizi Ouzou between the autumn of 2015 and the spring of 2016. Faecal samples were microscopically examined using the modified Ziehl-Neelsen acid-fast technique as screening method. Microscopy-positive samples were confirmed by a commercial coproantigen enzyme-linked immunosorbent assay (Bio-X Diagnostics). The identification of *Cryptosporidium* species and sub-genotypes in confirmed samples was conducted by PCR and sequence analyses of the small subunit ribosomal RNA (*ssu* rRNA) and the 60 kDa glycoprotein (*gp60*) genes of the parasite. Overall, 52.2% (240/460) of the investigated cattle tested positive to *Cryptosporidium* by microscopy. The infection was widespread in all 10 farms surveyed, but was significantly more prevalent in those from Blida in the central part of the country. Bovine cryptosporidiosis affected cattle of all age groups but with different outcomes. Pre-weaned (up to one month old) calves typically presented with diarrhoea, whereas older animals mostly harboured subclinical infections. The commercial ELISA used only detected 15.8% (38/240) of the samples that previously tested positive by microscopy, demonstrating a poor performance in field epidemiological surveys. Sequence analysis of the 29 isolates generated at the *ssu* rRNA loci confirmed the presence of four *Cryptosporidium* species including *C. parvum* (72.4%), *C. bovis* (13.8%), *C. andersoni*, (3.4%), and *C. ryanae* (3.4%). Two additional isolates (7.0%) could only be identified at the genus level. Eight out of the 21 isolates assigned to *C. parvum* were identified as sub-genotype IIaA16G2R1 at the *gp60* locus. *C. parvum* was almost exclusively found infecting pre-weaned calves, whereas *C. ryanae* and *C. andersoni* were only detected in asymptomatic animals. Bovine cryptosporidiosis is highly endemic in the surveyed area and represents a veterinary public health concern that should be adequately tackled by Algerian veterinary health authorities and policy makers.

1. Introduction

The enteric protozoan parasite *Cryptosporidium*, the ethiological agent of cryptosporidiosis, is a major diarrhoeal pathogen in humans and livestock with a global distribution (Ryan et al., 2014). In humans, cryptosporidiosis is the second cause of diarrhoea and death in children under five years of age after rotavirus (Striepen, 2013). In immunocompetent individuals the disease is self-limited, but it can be severe and life-threatening among immunocompromised subjects such as AIDS patients or those undergoing cancer chemotherapy or solid organ transplantation (Marcos and Gotuzzo, 2013). Cryptosporidiosis is also regarded as a significant cause of acute diarrhoea in farm ruminants affecting primarily neonatal animals (de Graaf et al., 1999). The clinical presentation of the infection vary from asymptomatic to deadly

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and, if improperly managed, can cause important economic loses associated to grow retardation, reduced productivity, and mortality (Santín, 2013).

The genus *Cryptosporidium* comprises at least 26 taxonomically valid species and over 40 genotypes of unknown species status characterized by marked differences in host range and specificity (Ryan et al., 2014). Of them, anthoponotic *C. hominis* and zoonotic *C. parvum* are responsible for the vast majority of human infections documented globally (Xiao and Feng, 2008). Similarly, cattle are a primary reservoir for four *Cryptosporidium* species, namely *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni*, and are considered a major contributor of *Cryptosporidium* oocysts in the environment via manure spreading in farmlands or grazing on watersheds (Xiao and Feng, 2008). Bovine infections seem to follow an age-related distribution pattern, with *C. parvum* primarily detected in pre-weaned calves, *C. bovis* and *C. ryanae* in asymptomatic animals aged between one and 6 months, and *C. andersoni* in older cattle (Santín et al., 2004, 2008).

Cryptosporidium is prevalent in both humans and domestic animals in Africa, although the molecular diversity and geographical distribution of the parasite is still largely unknown in many regions of the continent (Squire and Ryan, 2017). In Algeria, microscopy-based studies have documented the presence of *Cryptosporidium* infections in 0.1% to 30% of the human populations surveyed (Benouis et al., 2013; Hamaidi-Chergui et al., 2013). Using the same diagnostic method, bovine cryptosporidiosis has been reported at infection rates varying from 19% to 84% in a limited number of epidemiological investigations conducted in the provinces of Bejaia, Setif, and Souk Ahras (Ouchene et al., 2014), Annaba and Eltarf (Hocine et al., 2016), and Batna (Benhouda et al., 2017) in the North-eastern part of the country. Molecular information on the presence and relative frequency of *Cryptosporidium* species are also scarce, with few studies reporting infections by *C. bovis*, *C. ryanae*, *C. parvum*, and *C. andersoni* in cattle (Baroudi et al., 2017; Benhouda et al., 2017), by *C. baileyi* and *C. meleagridis* in poultry and wild birds (Baroudi et al., 2013; Laatamna et al., 2017), and by *C. erinacei*, *C. parvum*, *C. hominis*, and *C muris* in horses and donkeys (Laatamna et al., 2013, 2015). Although no molecular data from human isolates are currently available, taken together this information indicates that *Cryptosporidium* infections seem common in Algeria and transmission of the parasite may involve a number of different pathways. In order to clarify this epidemiological scenario, we present here novel data on the presence, molecular diversity and frequency of *Cryptosporidium* in neonatal calves from three geographically different Algerian regions. We used genotyping and subtyping tools for the molecular investigation of the obtained *Cryptosporidium* isolates.

2. Material and methods

2.1. Study herds and sample collection

A total of 20 dairy farms (herd size range 16–273 animals; age range two days to 18 months) belonging to private producers and with an unknown history of *Cryptosporidium* infection were selected and invited to participate in the study, of which 10 (50.0%) agreed to be enrolled. The farms belonged to the provinces of Aïn Defla, Blida, Sétif, and Tizi Ouzou and were geographically distributed into 3 regions as follows: region 1 included north-western Algeria ($n = 1$); region 2, north-central Algeria ($n = 2$); and region 3, north-eastern Algeria ($n = 7$) (Fig. 1). Farms were visited once during autumn of 2015 and spring 2016 and individual faecal specimens were collected from 460 cattle randomly selected for sampling. At least 5 g of faecal material were directly obtained from the rectum of each calf using a single pair of latex gloves and placed in screw-topped specimen containers. Cups were labelled with a unique identification number and accompanied by a form recording the date of sampling, the farm of origin, and the animal's sex, age, and breed. Information regarding animal housing, management, and feeding routines were also gathered and carefully recorded. Samples were shipped to the laboratory in refrigerated boxes and processed within 12 h of collection.

2.2. Microscopy

Microscopic examination for the presence of *Cryptosporidium* oocysts was conducted on faecal suspensions after formol-ether concentration (Allen and Ridley, 1970). Modified Ziehl-Neelsen acid-fast staining was performed on the obtained concentrates. Briefly, a drop of faecal suspension was placed on a glass slide to form a thin smear. Slides were fixed in methanol for 5 min and then flooded with carbol fuchsin for 1 h. After rinsing off in tap water, the slides were decolorized in 25% sulphuric acid for 60–90 s, washed, and counterstained with 5% malachite green for 10 min. The slide was rinsed with tap water, allowed to dry, and examined under a microscope at 400× and 1000× magnification.

2.3. Coproantigen enzyme-linked immunosorbent assay (CpAg-ELISA)

Cryptosporidium-positive samples at microscopic examination were subsequently confirmed using a commercially available test based on a two-site sandwich ELISA format (BIO K 346 ELISA, Bio-X Diagnostics, Rochefort, Belgium) on freshly preserved, unconcentrated faecal specimens following the manufacturer's recommendations. The assay relies on monoclonal antibodies directed against cell wall antigens of the parasite oocyst. Optical densities were measured at a wavelength of 450 nm in a 96-well microplate reader (Dialab, Neudorf, Austria). This kit has, according to the manufacturer, a diagnostic specificity and sensitivity of 90.9% and 97.1%, respectively. Samples that tested positive both by microscopy and ELISA were preserved in 70% ethanol and aliquots shipped to the Spanish National Centre for Microbiology, Majadahonda (Spain) for downstream molecular analyses.

2.4. DNA extraction and purification

Total DNA was extracted from ~200 mg of each faecal specimen using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified DNA samples (200 µL) were stored at 4 °C until PCR analysis.

2.5. Molecular detection, typing and sub-typing of Cryptosporidium spp. isolates

Faecal samples that tested positive for *Cryptosporidium* oocysts by any of the screening methods used were subsequently confirmed by a nested-PCR protocol targeting a 587-bp fragment of the small subunit (*ssu*) rRNA gene of the parasite (Tiangtip and Jongwutiwes, 2002). Amplification reactions (50 µL) consisted of 3 µL of DNA sample, 0.3 µM of each primer (CR-P1/CR-P2 in the primary reaction and CR-P3/CPB-DIAGR in the secondary reaction, respectively), 2.5 units of MyTAQ™ DNA polymerase (Bioline GmbH), and 10 µL of MyTAQ™ Reaction Buffer containing 5 mM dNTPs and 15 mM $MgCl_2$. Both PCR reactions were carried out as follows: one cycle of 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 s, 50 °C for 40 s and 72 °C for 1 min, concluding with a final extension of 72 °C for 10 min.

Cryptosporidium isolates identified as *C. parvum* at the *ssu* rRNA locus were subsequently investigated at the gene codifying for the 60 kDa glycoprotein (*gp60*) in order to ascertain the sub-type of the parasite. A nested-PCR protocol was used to amplify a 870-pb partial fragment of the *gp60* marker (Feltus et al., 2006). Briefly, 3 and 2 µL of template DNA were amplified in the first and second round of PCR, respectively, using 0.3 µM of each primer (AL-3531/AL-3535 in the primary reaction and AL-3532/AL-3534 in the secondary reaction, respectively). Amplification reaction mixes (50 µL) also contained 2.5 units of MyTAQTM DNA polymerase (Bioline GmbH), and 10 µL of MyTAQTM Reaction Buffer consisting of 5 mM dNTPs and 15 mM

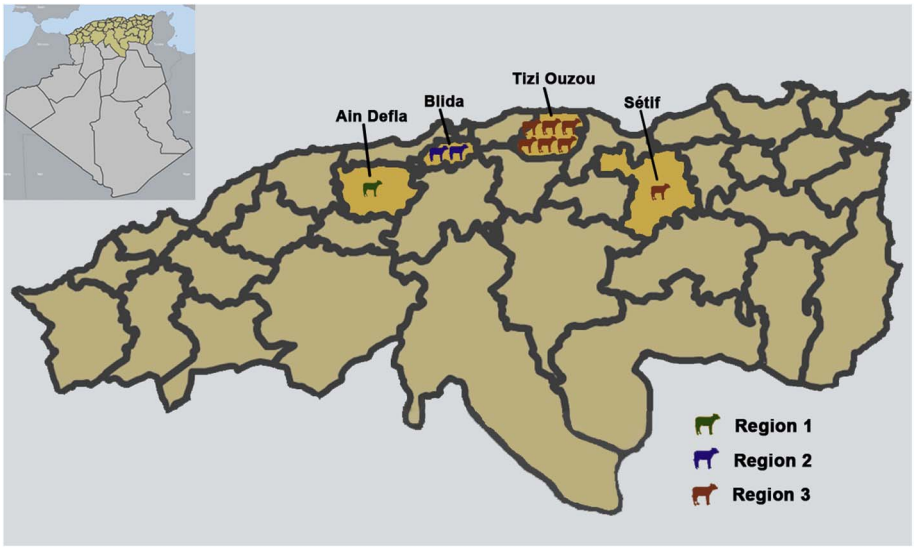

Fig. 1. Map showing the location and geographical distribution of the dairy farms investigated in the present study. The map of Input Administrative Unit Boundaries for Algeria was downloaded from the NASA Socioeconomic Data and Applications Center (SEDAC) at http://sedac.ciesin.columbia.edu/data/collection/groads/maps/gallery/search. Accessed on 21 May 2017.

Table 1
Cryptosporidim spp. infection rates determined by microscopic examination of cattle faecal specimens (n = 460) according to the farm and region of origin, Algeria, 2015–2016.

Region	Province	Farm code	Cattle at farm	Sampled cattle	Microscopy (+)	Percentage
North-western Algeria	Aïn Defla	1	273	120	60	50.0
North-central Algeria	Blida	2	30	30	30	100
		3	47	40	34	85.0
North-eastern Algeria	Sétif	4	16	16	16	100
	Tizi Ouzou	5	130	100	38	38.0
		6	30	26	13	50.0
		7	45	35	19	54.3
		8	38	25	14	56.0
		9	50	45	10	22.2
		10	27	23	6	26.1
Total			686	460	240	52.2

$MgCl_2$. Primary cycling conditions were as follow: 5 min at 94 °C followed by 35 cycles of 45 s at 94 °C, 45 s at 59 °C and 1 min at 72 °C, with a final extension of 72 °C for 10 min. The secondary PCR was similar to that described for the primary PCR step with the exception that the annealing temperature was 50 °C.

All PCR reactions were carried out on a 2720 thermal cycler (Applied Biosystems, California, USA). Laboratory-confirmed positive and negative DNA samples were routinely used as controls and included in each round of PCR. PCR amplicons were visualized on 2% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe nucleic acid staining solution (Conda). Positive-PCR products were directly sequenced in both directions using the internal primer set described above. DNA sequencing was conducted by capillary electrophoresis using the BigDye® Terminator chemistry (Applied Biosystems).

2.6. Data analyses

The chi-square test was used to compare *Cryptosporidium* infection rates in the bovine herds under study by age group and farm of origin. The same test was also used to ascertain the frequency of *Cryptosporidium*-associated diarrhoea according to the age group of the cattle. A probability (P) value < 0.05was considered evidence of statistical significance.

Raw sequencing data in both forward and reverse directions were viewed using the Chromas Lite version 2.1 sequence analysis program (http://chromaslite.software.informer.com/2.1/). The BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare nucleotide sequences with sequences retrieved from the National Center for Biotechnology Information (NCBI) database. Generated DNA consensus sequences were aligned to appropriate reference sequences using the MEGA 6 free software (http://www.megasoftware.net/) to identify *Cryptosporidium* species sub-types (Tamura et al., 2013).

For the identification of the phylogenetic inferences among the identified positive samples, a phylogenetic tree was inferred using the Neighbor-Joining method in MEGA 6. The evolutionary distances were computed using the Kimura 2-parameter method, and modelled with a gamma distribution. The reliability of the phylogenetic analyses at each branch node was estimated by the bootstrap method using 1000 replications. The sequences obtained in this study have been deposited in GenBank under accession numbers MF150290 to MF150295.

3. Results

The prevalence of bovine cryptosporidiosis in the three Algerian regions investigated was estimated at 52.2% (95% confidence interval: 47.6–56.7%) by microscopy, with *Cryptosporidium* oocysts being detected in 240/460 of the faecal samples examined (Table 1). The parasite was present in all 10 farms surveyed, where 67.1% (460/686) of cattle present at the time of the study were sampled. Marked differences in prevalence, ranging from 17% to 100%, were observed among the investigated farms. Farms located in north-central Algeria harboured significantly higher infection rates of bovine cryptosporidiosis than those in the north-western (χ^2 = 33.5; P < .00001) and north-eastern (χ^2 = 52.5; P < .00001) regions of the country.

Out of the 240 *Cryptosporidium* infections detected, 26.3% occurred in calves aged two to 15 days old, 22.1% in calves aged 16 to 30 days old, 37.1% in cattle aged > 1 month to 12 month old, and 14.5% in cattle older than 12 month. Regarding the presence of clinical

Table 2
Performance of a coproantigen enzyme-linked immunosorbent assay (ELISA) for the detection of *Cryptosporidim* spp. in cattle faecal specimens that tested positive to the parasite at microscopic examination (n = 240). Results by animal's age group and faeces consistency are indicated, Algeria, 2015–2016.

Age (days)	No. samples	Diarrhoeal samples			Non-diarrhoeal samples		
		n	+	%	*n*	+	%
2–15	63	52	16	30.8	11	3	27.3
16–30	53	16	11	68.7	37	3	8.1
> 30	124	6	3	50.0	118	2	1.7
Total	240	74	30	40.5	166	8	4.8

symptoms, 30.8% (74/240) of the animals with cryptosporidiosis presented with diarrhoea at the moment of the survey, with the remaining 69.2% (166/240) harbouring sub-clinical infections (Table 2). Diarrhoeic samples were significantly more frequently detected in infected calves in the 2–15 days ($\chi^2 = 117.9$; $P < 0.00001$) and 16–30 days ($\chi^2 = 21.9$; $P < 0.00001$) age groups than in older animals, respectively. The commercial CpAg-ELISA assay only confirmed the presence of *Cryptosporidium* oocysts in 15.8% (38/240) of the faecal samples that previously tested positive by microscopic examination. Positive CpAg-ELISA results were more often obtained in diarrhoeal samples from calves up to 1 month-old (Table 2).

Out of the 38 faecal specimens with a *Cryptosporidium*-positive result by both microscopy and ELISA, 76.3% (29/38) yielded amplicons of the expected (~480 bp) size at the *ssu* rRNA-PCR. Sequencing analyses allowed the identification of four *Cryptosporidium* species circulating in the surveyed calf population, including *C. parvum* (72.4%, 21/29), *C. bovis* (13.8%, 4/29), *C. andersoni*, (3.4%, 1/29), and *C. ryanae* (3.4%, 1/29). Two additional isolates (7.0%) were only identified at the genus level due to poor sequence quality (Table 3). The occurrence of these *Cryptosporidium* species followed a clear age-related pattern. *C. parvum* was the almost only species found in both symptomatic (91%) and asymptomatic (68%) calves in the 2–15 days age group, although a sub-clinical infection by *C. ryanae* was also identified in a 12-day-old, asymptomatic calf. *C. parvum* infections prevailed in calves in the 16–30 days age group too, with the exception of two (one symptomatic, one asymptomatic) calves infected by *C. bovis*. However, *C. parvum* infections were absent in calves older than 30 days, where *C. bovis* was the most prevalent (67%) species found. Additionally, a single infection by *C. andersoni* was found in an 18-month-old, asymptomatic heifer (Table 3). No apparent differences in the geographical distribution of *Cryptosporidium* infections were noted. Both *C. parvum* and *C. bovis* evidenced a rather wide distribution, being present in the three Algerian regions under study. *C. parvum* was present in all exploitations investigated except farms 1 and 3, whereas *C. bovis* was found in farms 1, 3, 8, and 9. *C. andersoni* was only reported in farm 1 (Western Algeria) and *C. ryanae* in farm 5 (Eastern Algeria). No *Cryptosporidium* isolates from farm 10 could be genotyped (Table 3).

Sub-genotyping data of the 27 *ssu* rRNA sequences unequivocally assigned to a given *Cryptosporidium* species are summarized in Table 4. The only isolate identified as *C. andersoni* contained a T insertion between nucleotides 633 and 634 of reference sequence AF093496 within a 494-bp (positions 528–1021) fragment. All four *C. bovis* isolates showed 100% homology to a 460-bp fragment (positions 311–770) of reference sequence AY741305. Interestingly, all 21*C. parvum* sequences generated in the present study were identical among them. Sequence alignment analyses with reference sequence AF112571 allowed the identification of a 498-bp fragment (positions 533–1030) with a number of single nucleotide polymorphisms (SNPs) including an ATTA deletion between nucleotides 688 and 691. Finally, the only isolate assigned to *C. ryanae* was identical to a 458-bp fragment (positions 314–771) of reference sequence EU410344. BLAST searches did not reveal the presence of novel genotypic variants.

The 21 isolates assigned to *C. parvum* at the *ssu* rRNA locus were subsequently assessed at the *gp60* locus in order to ascertain the sub-genotype(s) of the parasite involved in the infections. Overall, amplification products of the expected (~830 bp) size were obtained in 38.1% (8/21) of the tested isolates. All eight *gp60* sequences were confirmed as sub-genotype IIaA16G2R1. Seven of them were identical to GenBank sequence KF128739 isolated in a yak in China, whereas the remaining one differed from them by a single SNP (T to A) at position 377 of KF128739 and represented an unreported genotypic variant. No polymorphic sites (double peaks) were observed in any of the *ssu* rRNA or *gp60* sequences analysed. Fig. 2 shows the phylogenetic relationship among *C. parvum* sub-genotypes at the *gp60* locus produced by the Neighbour-Joining method. As expected, the two IIaA16G2R1 sequences generated in the present study grouped together in a well-defined cluster with previously reported bovine isolates belonging to the sub-genotype family IIa.

4. Discussion

Cryptosporidium, together with enterotoxic *Escherichia coli*, rotavirus and coronavirus, is a major contributor to the burden of diarrhoeal disease in neonatal calves globally (Foster and Smith, 2009). Neonatal morbidity and mortality due to cryptosporidiosis also represents a major economic concerns to the livestock industry (de Graaf et al., 1999). In addition, infected calves have been identified as important sources of environmental contamination with zoonotic *C. parvum*

Table 3
Distribution of *Cryptosporidium* species and sub-genotypes (n = 29) according to the age group, faeces consistency, and farm of origin of the infected cattle, Algeria, 2015–2016.

Age (days)	Diarrhoea	Farm code	Isolates (*n*)	Species	Relative frequency (%)	*gp60* sub-genotype (*n*)
2–15	Yes	4, 5, 8, 9	10	*C. parvum*	90.9	IIaA16G2R1 (4)
		4	1	*Cryptosporidium* spp.	9.1	Unknown
	No	5, 6	2	*C. parvum*	66.7	IIaA16G2R1 (1)
		5	1	*C. ryanae*	33.3	–
16–30	Yes	5, 7, 8	7	*C. parvum*	87.5	IIaA16G2R1 (3)
		9	1	*C. bovis*	12.5	–
	No	2, 5	2	*C. parvum*	66.7	Unknown
		8	1	*C. bovis*	33.3	–
> 30	Yes	1, 3	2	*C. bovis*	66.7	–
		4	1	*Cryptosporidium* spp.	33.3	Unknown
	No	1	1	*C. andersoni*	100	–

Table 4
Diversity, frequency, and main molecular features of *Cryptosporidium* isolates at the *ssu* rRNA locus in cattle, Algeria 2015–2016. GenBank accession numbers of representative sequences were provided.

Species	No. isolates	Reference sequence	Stretch	Single nucleotide polymorphisms	GenBank accession no.
C. andersoni	1	AF093496	528–1021	633_634insT	MF150290
C. bovis	4	AY741305	311–770	None	MF150291
C. parvum	21	AF112571	533–1030	A646G, T649G, 688_691delATTA, T693A	MF150292
C. ryanae	1	EU410344	314–771	None	MF150293

oocysts, a fact with serious public health implications (Wielinga et al., 2008). Given this scenario, accurate identification of cryptosporidiosis and *Cryptosporidium* species at the herd level is important not only to adopt adequate treatment and preventive measures to reduce *Cryptosporidium* burden on farm, but also to minimize the zoonotic risk involved (Hoek et al., 2008; Kinross et al., 2015; Suler et al., 2016).

In the present survey, one in two cattle were found infected by *Cryptosporidium*, a prevalence rate well in the range with those

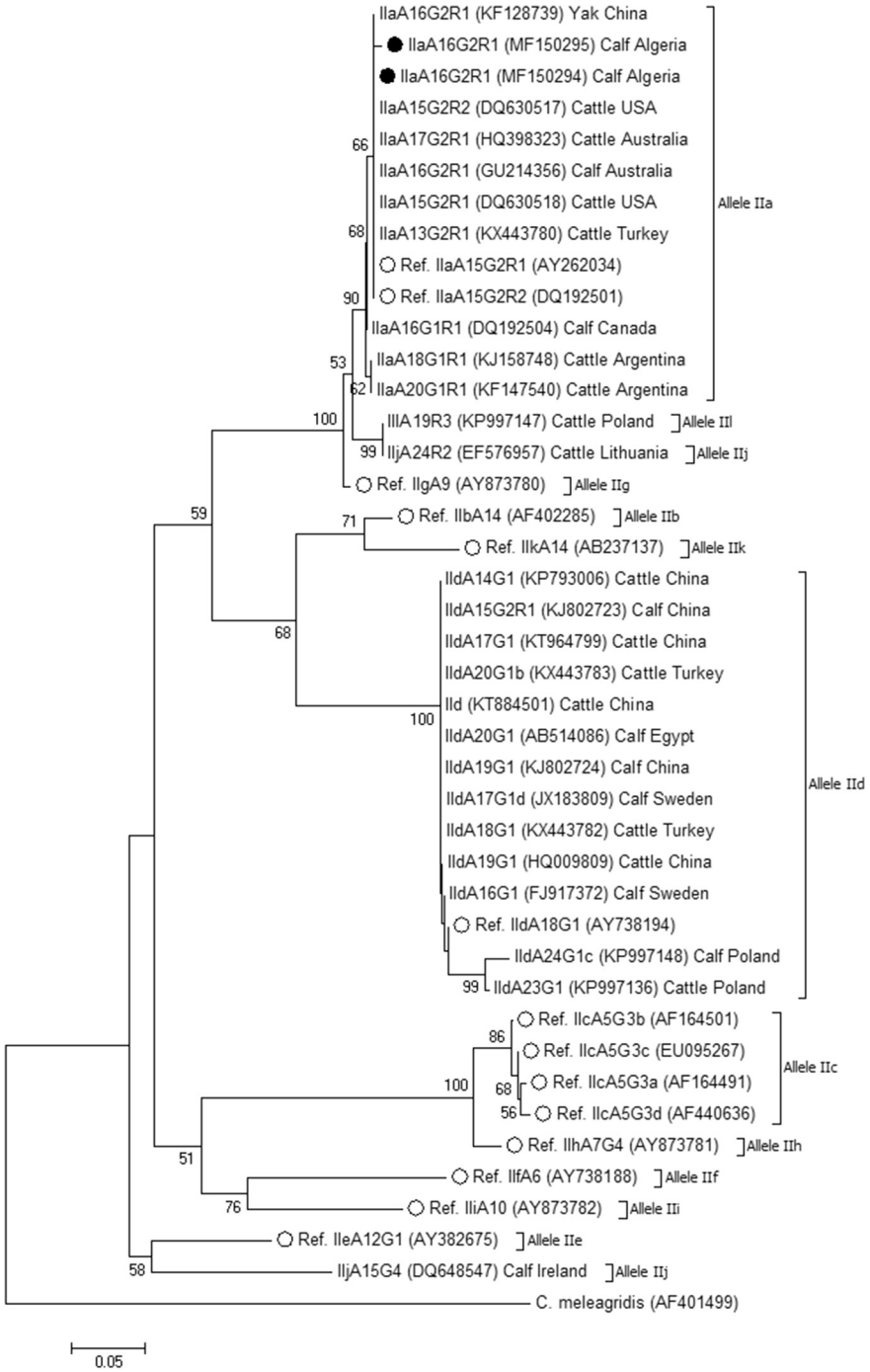

Fig. 2. Evolutionary relationships among *C. parvum* sub-genotypes at the *gp60* locus inferred by a Neighbor-Joining analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 iterations) is indicated next to the branches. Bootstrap values lower than 50% were not displayed. The evolutionary distances were computed using the Kimura 2-parameter method. The rate variation among sites was modelled with a gamma distribution (shape parameter = 2). Filled circles represent IIa sequences generated in this study. Open circles represent reference sequences extracted from GenBank. *C. parvum* sequences of bovine origin previously reported in other countries were included in the analysis for comparative purposes. *C. meleagridis* was used as outgroup taxa.

(23%–84%) previously reported in similar microscopy-based epidemiological surveys conducted in Algeria (Ouchene et al., 2014; Hocine et al., 2016; Benhouda et al., 2017). Using a variety of diagnostic methods, bovine cryptosporidiosis has also been documented, mainly in calves, at 32% in Egypt (Helmy et al., 2015), 29% in Madagascar (Bodager et al., 2015), 52% in Nigeria (Ayinmode et al., 2010), and 87% in Tunisia (Soltane et al., 2007). Despite the fact that herd prevalence was 100%, *Cryptosporidium* infections were significantly more common in farms located in north-Central Algeria. Overall, these findings strongly suggest that bovine cryptosporidiosis is endemic in the country, with spatial variations of prevalence probably reflecting differences in herd and farm management practices. Our results also revealed that *Cryptosporidium*-associated diarrhoea primarily affects preweaned (up to one month-old) calves, with older animals mostly harbouring sub-clinical infections. This is in agreement with previous studies showing that neonatal calves are highly susceptible to *Cryptosporidium* infections because of their immature immune system and high exposure to manure or equipment contaminated with oocysts of the parasite (e.g. Foster and Smith, 2009; Murakoshi et al., 2013; Björkman et al., 2015).

A puzzling finding was the poor performance delivered by the commercial CpAg-ELISA used in the present study, which was able to detect only a small fraction (16%) of the microscopy-positive samples. This very same test has been previously used in similar veterinary epidemiological surveys conducted in Iran (Badiei et al., 2011) and Iraq (Al-Robaiee and Al-Farwachi, 2014). Unfortunately, no other comparative diagnostic methods were simultaneously used in those investigations, making impossible to evaluate the actual diagnostic sensitivity of the CpAg-ELISA in field conditions. A number of commercial and in-house CpAg-ELISA tests have been developed for the detection of *Cryptosporidium* in faecal material from human (Garcia and Shimizu, 1997; Chalmers et al., 2011) or animal (Kuhnert-Paul et al., 2012; Ezzaty Mirhashemi et al., 2015) origin. When compared against PCR as gold standard, enzyme immunoassays typically achieved diagnostic sensitivities in the rage of 91–93%, significantly higher than those provided by the modified Ziehl-Neelsen microscopy (Chalmers et al., 2011; Kuhnert-Paul et al., 2012). Although some surveys have reported reduced capacity of ELISA to identify the presence of *Cryptosporidium* antigens in samples with low numbers of oocysts in human (Johnston et al., 2003) and animal (Ezzaty Mirhashemi et al., 2015) faecal specimens, this does not seem to be the case of our study, where a large proportion of the samples corresponded to symptomatic calves, expected to have high parasite burdens.

Bovine cryptosporidiosis is typically caused by four *Cryptosporidium* species, including *C. parvum*, *C. bovis*, *C. andersoni*, and *C. ryanae* (Xiao and Feng, 2008; Ryan et al., 2014), although *C. felis* (Cardona et al., 2015), *C. hominis* (Smith et al., 2005), *C. suis* (Fayer et al., 2006; Bodager et al., 2015), and *Cryptosporidium* pig genotype II (Langkjaer et al., 2007) have also been sporadically reported infecting cattle. Among them, *C. parvum* shows a marked host age-related susceptibility and has been identified as the most dominant *Cryptosporidium* species infecting pre-weaned calves, particularly by the subtype IIaA15G2R1, in different studies worldwide (Santín et al., 2004, 2008; Geurden et al., 2007; Wielinga et al., 2008; Rieux et al., 2013). This was indeed the case of our study, where *C. parvum* was responsible for the majority (84%) of the infections in calves in the 2–30 days age group. Interestingly all *C. parvum* isolates were assigned to IIaA16G2R1, a sub-genotype less frequently documented causing bovine infections but already described in cattle in Algeria (Baroudi et al., 2017), Belgium (Geurden et al., 2007), and Spain (Quilez et al., 2008), and in yaks in China (Mi et al., 2013). Human infections by IIaA16G2R1 have also been reported in clinical and paediatric populations in Democratic Republic of São Tomé and Principe (Lobo et al., 2014) and Iran (Sharbatkhori et al., 2015; Ranjbar et al., 2016), highlighting the zoonotic potential of this *C. parvum* sub-genotype. It is also worth mentioning that other members of the sub-type family IIa found infecting Algerian cattle include IIaA13G2R1 (Benhouda et al., 2017) and IIaA17G3R1 (Baroudi et al., 2017), whereas IIaA15G2R1 has not been identified in isolates of human or animal origin in the country to date.

In the present study *C. bovis* was, after *C. parvum*, the second most prevalent *Cryptosporidium* species circulating in cattle, being only identified in calves older than 15 days of age. This is contrast with the molecular data available in young calves (< 2 months) from the province of Batna in north-eastern Algeria (Benhouda et al., 2017), where *C. bovis* ($n = 14$) was the most common *Cryptosporidium* species found, followed by *C. ryanae* ($n = 6$) and *C. parvum* ($n = 4$). Additionally, we confirmed the presence of *C. ryanae* and *C. andersoni* in an asymptomatic calf and heifer, respectively. This is in line with previous surveys demonstrating that both species typically cause sub-clinical infections in cattle older than one month of age (Santín et al., 2004, 2008).

In conclusion, our results seem to indicate that bovine cryptosporidiosis is endemic in Algeria. *Cryptosporidium* infection greatly contributes to the burden of diarrhoeal disease in neonatal calves, very likely causing severe (although still unquantified) economic losses due to morbidity and mortality. Generated molecular data evidenced the presence of potentially zoonotic *C. parvum* sub-genotypes, a finding of public health relevance taking into account the extremely high environmental load of oocysts generated by calves at the peak of the infection. This situation highlights the importance of implementing proper farm management practices to effectively reduce the *Cryptosporidium* burden on affected farms.

Conflict of interest

None.

Ethical statement

This study was carried out in compliance with the national animal welfare regulations. Diagnostic veterinary procedures are not within the context of relevant EU legislation for animal experimentation (Directive 86/609/EC) and may be performed in order to diagnose animal diseases and improve animal welfare. Faecal samples were collected by trained personnel who ensured owners consent and caused neither harm nor suffering to the investigated animals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2018.02.005.

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