**INTRODUCTION**

The genus *Cryptosporidium* (Apicomplexa: Eucoccidiordia: Cryptosporiidae) is responsible of gastrointestinal illness in a wide variety of animals including humans (Caccio, 2005; de Graaf et al., 1999; Joachim, 2004). In calves, *Cryptosporidium* is one of the primary etiologic agents of neonatal calf diarrhoea (Nydam et al., 2001). Though one to three weeks old calves seem to be most susceptible, *Cryptosporidium* spp. has also been found yet in cattle over two years of age (Henriksen & Krogh, 1985). Dairy calves can excrete high numbers of oocysts for weeks, and there are indications that the disease can potentially reduce the growth performance of ruminants (Ralston et al., 2003) and cause high morbidity and sometimes high mortality rates in calves (Singh et al., 2006; Fayer et al., 1997; Olson et al., 2004a). A number of North American and European studies have shown *Cryptosporidi-**

**MATERIAL AND METHODS**

**Faecal specimens**

Samples were collected in 2000 from one dairy farm localised in the Sfax district (center east of Tunisia). The farm was selected taking into consideration proximity to the university and farmer cooperativeness. Thirty calves were surveyed from the newborn to the one month age. Stools were collected directly from the rectum, daily for the first week, every two days during the second week and every three days during the third and fourth weeks. Sixteen stool spe
cimens were, therefore, obtained from each calf (a total of 480 calves stools specimen was examined). A calf was considered infected with *Cryptosporidium* if oocysts were microscopically observed in at least one stool specimen. The consistency of the stool was recorded as liquid, soft or normal, the two former being considered as diarrhoea. Twenty five samples of soil (including areas where infected calves were reared) were also collected from the farm. The faecal specimens were stored at 4°C in a 2.5 % aqueous potassium dichromate solution until examination.

**Microscopic examination**

To diagnose the *Cryptosporidium* presence in calves, a fraction of faecal specimens was concentrated by the conventional Formalin-ether sedimentation technique (Ritchie) (Young *et al.*, 1979). Smears of emulsified faecal pellets were then stained by modified Ziehl Neelsen technique (Henriksen & Pohlenz, 1981) and screened for *Cryptosporidium* oocysts by microscopic examination at 1000× magnification.

**Statistical analysis**

A Chi-square test using a 95 % confidence interval was used to study the relationship between the two variables diarrhoea and *Cryptosporidium* detection in newborn calves.

**Molecular analysis for genotyping**

*Cryptosporidium* genotyping was done subsequently only for seven isolates of calves. For DNA extraction, oocysts contained in stools were ruptured by using three freeze-thaw cycles (liquid nitrogen, 3 min, and 56°C, 3 min) in a lysis buffer (Tris 10mM; EDTA 0.1 mM; SDS 1 %). Proteinase K was added at a final concentration of 0.2 mg/ml and an overnight digestion at 55°C was performed. DNA was then extracted by using phenol and chloroform-isoamyl alcohol. DNA was precipitated by ethanol in presence of sodium acetate.

Crude DNA was purified using the DNA Clean Up Kit (Promega, Madison, Wis.). An additional polyvinylpyrrolidone (PVP, Sigma-Aldrich) treatment of the DNA was finally performed to eliminate all polymerase chain reaction (PCR) inhibitors. For molecular identification, a fragment of the 18S rRNA gene was amplified by nested PCR using primers reported by Xiao *et al.* (2001). Amplified products were sequenced by using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). In addition to this genotyping technique, DNA amplification and restriction fragment length polymorphism (RFLP) at the Laxer locus were performed as previously reported (Guyot *et al.*, 2002). The nucleotide sequences generated in this study has been deposited in GenBank under accession number EF158462.

**Results**

All cattle faecal samples (n = 480) were collected from 30 young calves bred in the same farm. The *Cryptosporidium* overall prevalence was 86.7 % (26/30). The oocyst excretion was first detected five days after birth with a prevalence of 10 % (Fig. 1). The percentage of calves infected increased up to 70 % at the age of 13 days and then decreased to reach 10 % at the age of 28 days. The calf’s diarrhoea was associated (P < 10⁻⁷) with the *Cryptosporidium* infection (Table I). 99 (20.6 %) of the samples had faecal scores indicative of diarrhoea. Of these diarrhoeic samples, 55 (55.5 %) were shedding *Cryptosporidium* oocysts. In

![Fig. 1. – Frequency distribution of calves infected with *Cryptosporidium* by age.](image-url)
contrast, only 23.3 % of the 381 non-diarrhoeic samples were shedding oocysts. No oocyst was detected in samples collected from the environment (n = 25) including areas where infected calves were reared. The molecular-based analysis concerned seven Cryptosporidium isolates. DNA was amplified by nested PCR and the resulting fragment of the 18s rRNA gene was sequenced. Sequences were identified as C. parvum. The Cryptosporidium DNA diagnostic fragment characterized by Laxer et al. (Guyot et al., 2002, Laxer et al., 1991) was amplified by PCR. For each isolate, a single amplicon was produced. In agreement with the genotyping of the 18S rRNA gene, C. parvum was identified in the seven calves. Moreover, all C. parvum were of the L1-subgenotype (Fig. 2).

DISCUSSION

The results of this study shows that Cryptosporidium infection occurred in calves in Tunisia, as also found in numerous similar studies in other countries. This is the first known study to estimate the prevalence of Cryptosporidium in calves in a country of North Africa. Since most dairy cattle become infected in the first month of life (Wade et al., 2000; Fayer et al., 1997), our sampling scheme was directed to target this subpopulation, increasing the chances of case detection. The prevalence of infection in newborn calves (86.7 %) reported in this study may have varied from some of the previous studies for a number of reasons. With the intermittent shedding pattern of the parasite, it is possible that with only one sample that the prevalence may have been somewhat underestimated (Buret et al., 1990; Ralston et al., 2003). Longitudinal studies have demonstrated that following animals over time will affect prevalence rates considerably. For example, in one longitudinal study, the prevalence varied with month of the year and animal age (Huëtink et al., 2001). The results of present study are in conformity to O’Handley et al. (1999), who described 100 % cumulative Cryptosporidium infection rates in dairy calves. They examined every-other-day 20 calves on one farm only. In this study, the examined dairy calves were infected from the first week of age. These results are consistent with the data of Fayer et al. (1998) and O’Handley et al. (1999), who found that calves usually became infected with Cryptosporidium between one and four weeks of age. No infection of Cryptosporidium was found until four days after birth, as Kvac et al. reported as well (2006).

Seventy percent of the 13-day-old calves were found infected with Cryptosporidium (Fig. 1). This corresponds to the first peak of cryptosporidiosis in calves that appears before the weaning. Genotyping could have been performed for samples from seven of these calves and C. parvum (bovine genotype) was identified in all cases. These results are in agreement with the work of Santin et al. (2004) in which C. parvum is shown to constitute 85 % of the Cryptosporidium infections in pre-weaned calves during the first peak of cryptosporidiosis but only 1 % of the Cryptosporidium infections in post-weaned calves, during the second peak of infection. Infection with C. parvum in neonatal calves was associated with diarrhoea. In the present study, 55.5 % of diarrheic and 23.3 % non-diarrheic samples were shedding oocysts. Lise et al. (2005) similarly found that 40.6 % calves were shedding C. parvum oocysts at the time of sampling and 50.5 % of diarrheic and 23.5 % non-diarrheic calves were shedding oocysts. In this study, no oocysts were recovered from soil samples, including areas where infected calves were reared. Although Barwick et al. (2003) reported that the environmental contamination of farms with oocysts was insufficient to account for the high levels of infection seen in cattle, contaminated soil is considered to be a sufficient infection source for newly introduced animals. Other studies support the importance of calf-to-calf contact in transmission (Xiao et al., 1993; O’Handley et al., 1999; Wade et al., 2000; Becher et al., 2004). The present work is the first report on Cryptosporidium occurrence in calves in Tunisia associating molecular characterization of few parasite isolates. Since C. parvum from cattle can infect humans, further studies with genotyping of human and animal isolates are necessary to evaluate the public health significance and give further insights into the epidemiology of the infection in Tunisia.
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REFERENCES


KVAČ M., KOUBA M & VITOVCE J. Age-related and housing-dependence of Cryptosporidium infection of calves from dairy and beef herds in South Bohemia, Czech Republic. Veterinary Parasitology, 2006, 137, 202-209.


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