CRYPTOSPORIDIUM PARVUM (EUCOCCIDIORIDA: CRYPTOSPORIIDAE) IN CALVES: RESULTS OF A LONGITUDINAL STUDY IN A DAIRY FARM IN SFAX, TUNISIA

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Summary:

A longitudinal study was undertaken to determine the prevalence of *Cryptosporidium* in a dairy farm in Sfax, Tunisia. 480 faecal samples were obtained from 30 calves under one month of age. All faecal samples were analysed for *Cryptosporidium* oocysts by microscopic examination of smears stained by modified Ziehl Neelsen technique. The parasite was detected in 26 calves (86.7 %). Infection was significantly associated with diarrhoea. A molecular characterization, performed in seven calves, confirmed that isolates were *C. parvum*. This work is the first report on *Cryptosporidium* in calves in Tunisia.

KEY WORDS : *Cryptosporidium*, Apicomplexa, prevalence, calves, genotyping, Tunisia.

Résumé : *CRYPTOSPORIDIUM* (APICOMPLEXA: EUCOCCIDIORIDA: CRYPTOSPORIIDAE) CHEZ LES VEAUX : RÉSULTATS D'UNE ÉTUDE LONGITUDINALE DANS UNE FERME LAITIÈRE À SFAX, TUNISIE

Une étude longitudinale a été réalisée pour déterminer la prévalence de Cryptosporidium *dans une ferme laitière à Sfax, Tunisie. 480 échantillons fécaux ont été obtenus à partir de 30 veaux d'âge inférieur à un mois. Tous ces échantillons ont été analysés par examen au microscope des frottis colorés par la technique de Ziehl Neelsen modifiée pour la détection des oocystes de* Cryptosporidium. *Le parasite a été détecté chez 26 veaux (86,7 %). L'infection a été significativement associée à la diarrhée. La caractérisation moléculaire, réalisée pour sept veaux, a confirmé que les isolats ont été* C. parvum. *Ce travail est le premier rapport sur* Cryptosporidium *chez des veaux en Tunisie.*

MOTS CLÉS : Cryptosporidium, *Apicomplexa, prévalence, veau, génotypage, Tunisie.*

INTRODUCTION

The genus *Cryptosporidium* (Apicomplexa: Eucoccidiorida: 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 *Cryptosporidium* to be highly prevalent in dairy calves with infection rates as high as 100 % in some herds, and have also demonstrated an association between parasite infection and diarrhoea and significant production losses (de Graaf *et al*., 1999; O'Handley *et al*., 1999; Huetink *et al*., 2001; Olson *et al*., 2004b). Up to now, no study has been carried out on the prevalence of *Cryptosporidium* in cattle in Tunisia. In light of the veterinary importance, causation of production losses and its zoonotical potential, more knowledge about the prevalence of the parasite was needed. The objective of the present study was to determine the prevalence of *Cryptosporidium* in calves in a dairy farm in Tunisia.

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MATERIAL AND METHODS

FAECAL SPECIMENS

Stool specimens 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 chloroforme-isoamylic 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 a*l. (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

Number of faecal samples	Diarrhoeic	Normal	Total
Positive for *Cryptosporidium*	55	89	144
Negative for *Cryptosporidium*	44	292	336
Total	99	381	480

Table I. – Cross tabulation of faecal consistency and detection of *Cryptosporidium* oocysts.

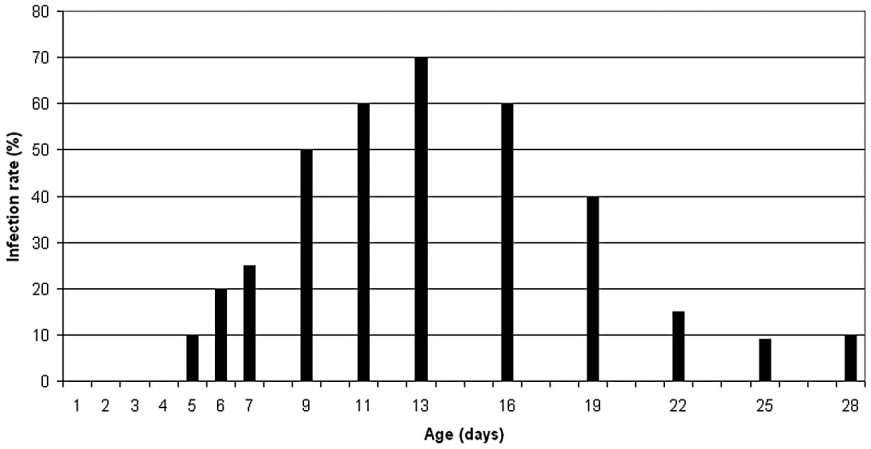

Fig. 1. – Frequency distribution of calves infected with *Cryptosporidium* by age.

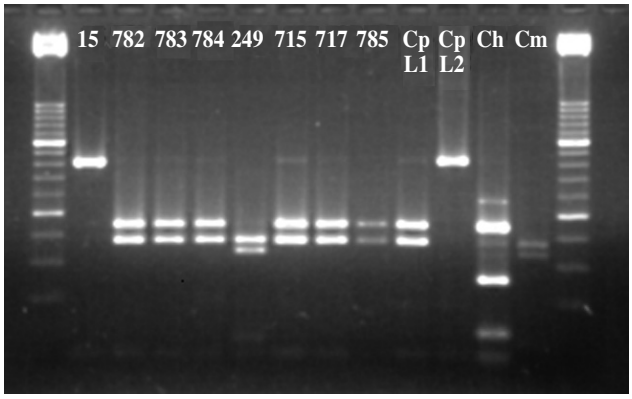

Fig. 2. – Ethidium bromide-stained agarose gel of the *Cryptosporidium* Laxer fragment after triple digestion with *Muv*I, *Mlu*I and *Bpm*I. Genotyped isolates were six of the seven calf-derived isolates (782, 783, 784, 715, 717, 785) (isolates 15 and 249 were a human and broiler chicken-derived isolates respectively). Isolate controls were *C. parvum* subgenotype L1 (CpL1), *C. parvum* subgenotype L2 (CpL2), *C. hominis* (Ch) and *C. meleagridis* (Cm). Size markers were DNA molecular weight marker XIII (Roche).

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 (Huetink *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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